University of Alberta

BACTERIAL ATTACK ON ALIPHATIC SULFIDES AND RELATED COMPOUNDS REPRESENTING THE SULFUR GROUPS IN HEAVY CRUDE OIL

by

©

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TO SEAN AND TO MY PARENTS

Abstract

Selective cleavage of the aliphatic sulfide bonds in asphaltenes decreases their molecular weight and may achieve the viscosity reduction required for transportation of heavy oils by pipeline. Enrichment cultures were used to isolate bacteria active towards model aliphatic sulfur compounds. The bacterium *Rhodococcus* sp. strain JVH1 was then studied as a potential biocatalyst for viscosity reduction.

Thirty-one bacterial strains isolated from enrichment cultures were able to transform model aliphatic sulfides. Three types of attack were identified: alkyl chain degradation, allowing use as a carbon source; non-specific sulfur oxidation; and sulfur-specific oxidation and carbon-sulfur bond cleavage, allowing use as a sulfur source. *n*-Octyl sulfide degradation proceeded through terminal oxidation followed by β-oxidation reactions. Utilization of benzyl sulfide and 1,4-dithiane as sulfur sources was regulated by sulfate, indicating sulfur-specific activity rather than non-specific oxidation. Several isolates were able to use dibenzothiophene as a sulfur source, and this was the preferred organic sulfur substrate for one isolate. The use of commercially available alkyl sulfides in enrichment cultures therefore gave isolates that followed a range of metabolic pathways, not just sulfur-specific attack.

JVH1 used a range of aliphatic sulfur compounds as sulfur sources for growth, as well as benzothiophene and alkylbenzothiophenes. JVH1 exhibited selectivity between some organic sulfur compounds. Selectivity was not observed when multiple sulfur compounds were provided in a second liquid phase, most likely due to the effects of partitioning. Inorganic sulfate repressed desulfurization of aliphatic and aromatic sulfur compounds. High salt concentrations and the addition of sulfate analogues did not alleviate this repression. The use of resting cell cultures may be useful for avoiding the regulatory effect of sulfate without genetic modification. JVH1 was able to degrade alkanes as carbon sources, and the degradation of *n*-hexadecane was not subject to catabolite repression. JVH1 grew with Lloydminster heavy crude oil as a sulfur source, resulting in loss of added benzyl sulfide and of native *n*-alkanes from the oil. Conversion of sulfur species was not sufficient to result in viscosity reduction. Instead, an increase in the viscosity of the oil was measured, attributable to oxidation of the alkanes and other oil components.

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List of Abbreviations

Compounds

HMN	2,2,4,4,6,8,8-Heptamethylnonane
PFPP-acid	3-Pentafluorophenylpropanoic acid
PFPP-OH	3-Pentafluorophenylpropan-1-ol
PFPS	*bis*-(3-Pentafluorophenylpropyl) sulfide
PFPSO	*bis*-(3-Pentafluorophenylpropyl) sulfoxide
$PFPSO_2$	*bis*-(3-Pentafluorophenylpropyl) sulfone

Growth media

½PCA	Half-strength Plate Count Agar
B+N8P	A mineral medium with extra buffering
B+NP	A mineral medium
BHMV	Modified Bushnell-Haas medium (a mineral medium with trace metals and vitamins)
PCA	Plate Count Agar
SFMM	Sulfur Free Mineral Medium
SFMM+1% glycerol	SFMM with 1 % glycerol (w/v)
SFMM+2Ac	SFMM with 2.0 g $CH_3COONa\cdot 3H_2O$ per litre
SFMM+4Ac	SFMM with 4.0 g $CH_3COONa\cdot 3H_2O$ per litre
S-free	Sulfur-free B+NP
S-free +2Ac	S-free with 2.0 g $CH_3COONa\cdot 3H_2O$ per litre
TSB	Trypticase Soy Broth

Analytical techniques and assays

BATH	Bacterial Adhesion to Hydrocarbons
CGA	Comparative Growth Assay
EI	Electron impact
FID	Flame ionization detection
FPD	Flame photometric detection
FTIR	Fourier transform infrared
GC	Gas chromatography (combined with FID, FPD, MS, FTIR)
MS	Mass spectrometry (combined with EI, NH_4^+ CI)
NH_4^+ CI	Ammonium chemical ionization
OD_{600}	Optical density at 600 nm

Other nomenclature

ANOVA	Analysis of Variance
HSD	Honestly Significant Difference (used in Tukey's test and Tukey-Kramer test)
K_{ow}	Octanol-water partition coefficient

1. Introduction

1.1 Background

The chemical goals of heavy oil upgrading encompass molecular weight reduction of residue fractions to distillate materials, hydrogenation to increase the hydrogen to carbon (H/C) ratio, and removal of heteroatoms, in particular sulfur and nitrogen [51]. The potential scope of biological oil upgrading can be defined more broadly, to include all activities which make the material easier to produce and transport, as well as the chemical changes which increase the value of the oil. These activities could therefore be applied to *in situ* treatment, production, transportation, and processing of crude oils. Five key areas of heavy oil upgrading where biological treatment could have an impact are viscosity reduction, composition improvement, deposition control, de-emulsification, and naphthenic acids removal (reviewed in [84]). In contrast to the available chemical processes, biological processing may offer less severe process conditions and higher selectivity to specific reactions.

The focus of this thesis is the investigation of biologically catalysed chemical reactions with the potential to reduce the viscosity of heavy oils. Heavy oils are currently diluted with light hydrocarbons to reduce viscosity and allow transportation by pipeline to processing facilities. Natural gas condensate is typically used as the diluent, but the supply of this material is insufficient for current production of heavy oil and bitumen and the cost is high. The production of heavy oil is expected to increase further over the next several years; therefore, alternate diluents or field upgrading technology to reduce viscosity is critically needed.

Experimental data show that the viscosity of oil is correlated with the average molecular weight of the material, and also increases with the asphaltene content (weight %) [27, 132]. Reducing the molecular weight of the asphaltene fraction could therefore contribute to a reduction in the viscosity of the oil. The key features of asphaltene molecules are polycyclic aromatic groups linked together by aliphatic chains and bridges of various lengths. Sulfides, esters, and ethers have all been identified as linking structures in these bridges [124]. Selective chemical cleavage of the aliphatic sulfide bonds in asphaltenes decreases their molecular weight [124] and may achieve the viscosity reduction required for transportation by pipeline. Bacterial cleavage of these carbon-sulfur bonds should achieve the same effect.

Aliphatic sulfides have not been studied in oil desulfurization research. The relatively low carbon-sulfur bond strength results in easy cleavage of these bonds under thermal treatment, compared to thiophenic sulfur, which is only removed during catalytic hydrotreating [51]. Research on biological desulfurization of oil has therefore focused on the recalcitrant thiophenic compounds, with dibenzothiophene as the primary model compound. Several bacteria selectively remove sulfur from dibenzothiophene, leaving the carbon structure intact as 2-hydroxybiphenyl [77, 120, 133]. The mechanism of desulfurization is known as the "4S" pathway, first identified in *Rhodococcus erythropolis* strain IGTS8 (ATCC 53968). Biological removal of aliphatic sulfides may occur through an analogous pathway, where the sulfur atom is oxidized and released as sulfite. This mechanism should be sufficient for molecular weight and viscosity reduction in heavy oil.

1.2 Research approach

This research project was based on two hypotheses: bacteria exist that possess enzymes with sulfur-specific carbon-sulfur bond cleavage activity towards aliphatic sulfide groups; and these bacteria are suitable candidates for the development of a biological upgrading technology for reducing the viscosity of heavy crude oil. The two main aspects of this project were therefore the identification of promising bacterial strains, and the characterization of those strains in the context of the biological upgrading application.

Crude oil is a complex mixture of aliphatic and aromatic hydrocarbons, as well as heteroatomic species, and biodegradation of the different crude oil components is well known for many bacterial strains (reviewed in [161]). Model compounds were therefore selected to represent the aliphatic sulfide bridges in asphaltenes, to allow identification and study of the very specific desired reactions. Pure cultures were also desirable, to limit the potential degradative activity of the cultures towards crude-oil-type molecules. Enrichment cultures were used to select for and isolate pure cultures with activity towards the model aliphatic sulfides.

Bacterial isolates were characterized with respect to their substrate ranges for sulfidic and thiophenic sulfur compounds, selectivity between different organic sulfur compounds, the effect of sulfate on desulfurization, and their ability to degrade hydrocarbons. The activity of one isolate towards crude oil was also investigated. These properties were selected not solely for scientific interest, but as being the most important concerns for the development of the upgrading application. Well-defined and controlled laboratory experimental conditions, rather than field conditions, were used to identify the specific capabilities of the isolates studied. While acknowledging that genetic modification could be essential to achieve a suitable biocatalyst for biological upgrading, the aim of this research was to examine the potential of the wild-type microorganisms in order to properly define, and hopefully limit, the need for such modifications.

1.3 Specific research objectives and thesis overview

The overall goal of this project was to study bacteria with selective, sulfur-specific activity towards aliphatic sulfides in the context of a biological upgrading process for reducing the molecular weight and viscosity of heavy crude oil. This work was done in two phases, with specific objectives as follows:

1. To establish enrichment cultures selective for degradation of model compounds representing the aliphatic sulfide bridges found in asphaltenes, and to identify bacterial isolates from the enrichment cultures that exhibit sulfur-specific carbon-sulfur bond cleavage. The following approach was taken (presented in Chapter 4):
    a. Select appropriate model compounds.
    b. Establish enrichment cultures with model compounds serving as the sole sulfur or carbon source for growth.
    c. Develop appropriate screening experiments to identify active enrichment cultures, as well as isolates from the enrichment cultures with activity towards the model compounds.
    d. Characterize promising isolates, including strain identification, substrate ranges for organic sulfur compounds and hydrocarbons, metabolite

formation from model compounds, and response of the isolates to inorganic sulfate.

2. To explore the potential of a genetically unmodified microorganism for biocatalytic upgrading to reduce crude oil viscosity, and to show whether naturally occurring biological control strategies can be exploited to achieve desired characteristics. Work in this phase of the project focused on *Rhodococcus* sp. strain JVH1 [160], and followed four avenues of investigation:
    a. Determine the substrate range and selectivity of the organism for model organic sulfur compounds and identify oxidation products (Chapter 5).
    b. Test the activity of the selected isolate towards model oils (Chapter 5) and whole crude oil (Chapter 6).
    c. Show whether inorganic sulfate has a negative effect on the desulfurization of organic sulfur compounds, and test different approaches to circumvent this effect (Chapter 7).
    d. Check whether hydrocarbons (alkanes and polycyclic aromatic hydrocarbons) are degraded as carbon sources by the isolate, and test possible means of suppressing this activity (Chapter 5).

2. Literature review [*]

2.1 Viscosity of heavy crude oil, relation to molecular composition and structure, and prospects for biological upgrading

2.1.1 Viscosity correlations

The viscosity of heavy oil is a result of interactions among the heaviest molecules in the oil, the asphaltenes. These interactions include entanglement of the alkane chains [51] as well as more ordered interactions between the aromatic clusters leading to structure formation throughout the oil [132]. One potential biotechnological approach to viscosity reduction is emulsification of the oil using bioemulsifiers such as emulsan (reviewed in [7]).

Breaking the asphaltenes into smaller molecules should also reduce molecular interactions leading to a reduction in viscosity. Experimental data show that the viscosity of oil is correlated to the average molecular weight of the material. Figure 2.1A shows data compiled from different sources, including whole oils, bitumen, distillates, and residues. The observable trends are towards higher viscosity in heavier samples and towards lower viscosity at higher temperatures. The scatter in Figure 2.1A indicates that there are factors involved other than molecular weight and temperature. Some published models include properties such as specific gravity to account for this variability (for example [134, 166]). Although viscosity models fit the data used to generate them, they are often difficult to extend to other samples due to these other contributing effects. The general correlation to molecular weight appears to be sound and can be used as a basis for further analysis of viscosity.

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[*] Portions of this chapter have been published:
Kirkwood, K.M., Foght, J.M., and Gray, M.R. 2004. *In* R. Vazquez-Duhalt and R. Quintero-Ramirez (eds.), Petroleum Biotechnology: Developments and Perspectives. Studies in Surface Science and Catalysis 151:113-143.

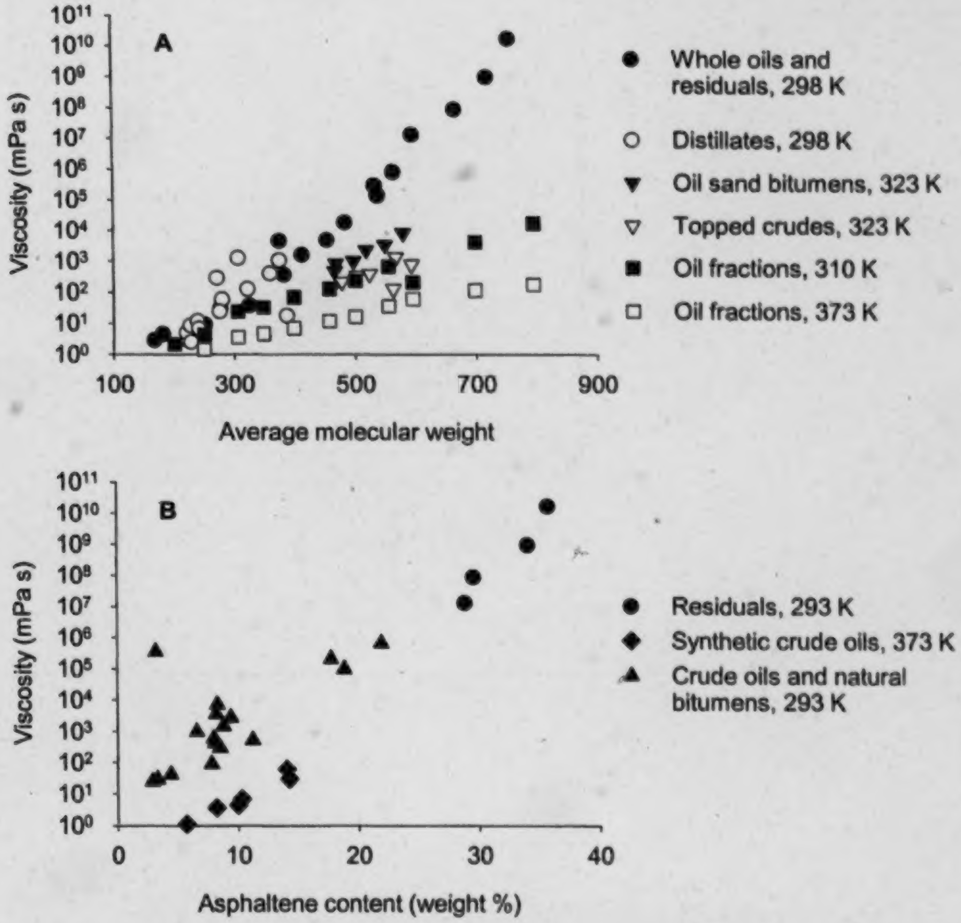


Figure 2.1. **Viscosity data for whole oils, residuals, and distillates [27], oil sand bitumens and topped crudes [166], oil fractions [134], synthetic crude oils [170], and crude oils and natural bitumens [132] showing correlation to (A) average molecular weight and (B) asphaltene content. (Analysis temperatures are indicated in the legends.)**

The correlation of viscosity and molecular weight indicates that there should also be a correlation between the viscosity and the fractional composition of the oil. Figure 2.1B shows that viscosity increases with the asphaltene content (weight %), the asphaltenes being among the heaviest molecules in the oil. This type of correlation has been used to formulate viscosity models based on logarithmic mixing rules, assigning "pseudo-viscosities" to the different fractions of the oil and applying a weighting factor to each one [170, 171].

2.1.2 Asphaltene structure

Because asphaltene content is strongly correlated to the viscosity of heavy oils, an understanding of asphaltene structure is necessary to develop a viscosity reduction process. Asphaltenes, however, are not classified by structure, but are defined as a solubility class, including material that is soluble in toluene but not in *n*-pentane (or alternatively *n*-heptane). There are two different views on the molecular structure of asphaltenic material. The first represents asphaltenes as having a single large condensed polycyclic aromatic core, with aliphatic chains attached on the periphery (Figure 2.2A) [53, 149, 172, 173]. This type of structure, however, does not account for all of the physical and chemical properties of asphaltenes [50]. The second representation describes asphaltenes as having multiple smaller polycyclic aromatic cores (2-4 rings) linked by aliphatic bridges of various lengths (Figure 2.2B) [5, 125, 151-153]. This type of structure accounts for the observed reactivity of asphaltenes [50].

Sulfides, ethers, and esters have been identified as common linking structures in the aliphatic bridges found in asphaltenes [124]. Nickel boride desulfurization was used to specifically cleave aliphatic sulfide bonds in two asphaltene fractions, giving a 4-fold reduction in the molecular weight of the higher molecular weight fraction. Aliphatic sulfides were therefore involved in the linking structures of the molecules, including linkages between aromatic cores and to smaller structures like alkanes and steranes. Bacterial cleavage of these carbon-sulfur bonds should achieve the same effect.

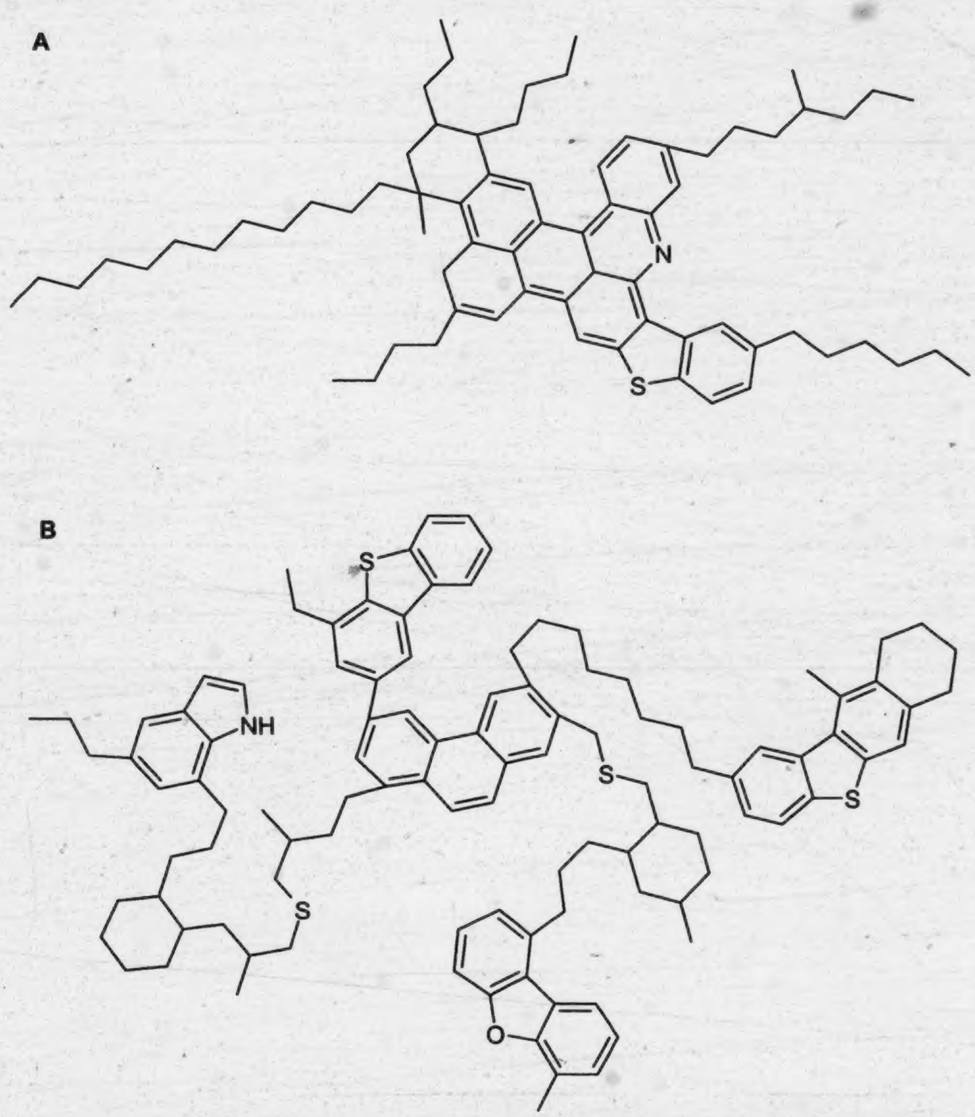


Figure 2.2. Representative models of asphaltene molecules showing either (A) a single large condensed polycyclic aromatic core [53] or (B) multiple smaller polycyclic aromatic cores with aliphatic bridges [5].

The total sulfur content of asphaltenes includes the sulfide bridges (aliphatic), cyclic sulfides (aliphatic heterocycles, found as substituted thiolanes and thianes [123]), and thiophenic sulfur (aromatic sulfur heterocycles). Only cleavage of the sulfide bridges leads to a reduction in molecular weight, because removal of the cyclic sulfides and thiophenes leaves the carbon backbone intact.

These large molecules are problematic for biological transformation. Transformation rates are limited by the mass transfer of target molecules to the biocatalyst and, in the case of whole cells, across the cell membrane (reviewed in [19]). Interfacial mass transfer can be improved through emulsification, increasing the interfacial contact area, however emulsification is of limited value in overcoming the barrier of transport into biological cells unless appropriate uptake mechanisms are available. Despite these difficulties, there is evidence in the literature for bacterial transformation of complex, high molecular weight substrates (some of which are discussed in Section 2.2.3).

2.2 Biological sulfur requirements and biodesulfurization

2.2.1 Sulfur limitation and sulfate repression

Sulfur is an essential element for bacterial growth. It is found in proteins, as part of the amino acids cysteine and methionine, as well as a variety of enzyme cofactors and vitamins such as biotin, thiamine, and coenzyme A. The total concentration of sulfur is low in cells, typically accounting for around 1 % of the dry weight of a bacterial cell [74]. The requirements for growth are correspondingly small; a C:N:P:S molar ratio of 700:80:3:1 has been recommended to support aerobic, heterotrophic bacterial growth [28]. Bacteria typically use sulfate as the preferred inorganic sulfur source. Sulfate is transported into the cell, where it is reduced to sulfite and sulfide before incorporation into cysteine [74].

Because a requirement for sulfur exists, it follows that a condition of sulfur starvation can exist, where metabolizable sulfur compounds are not available in sufficient quantities to support growth. Sulfate limitation is rare, but can occur, for instance in soils, where 50 % or more of the sulfur may be found in sulfate esters and sulfonates, and less than 5 % as free sulfate [74]. The ability to use sulfur sources other than sulfate may be essential for survival in certain soil environments where carbon and nitrogen sources are relatively abundant, but sulfur is bound in organic forms [111]. Survival under conditions of sulfur limitation would require physiological changes in the cell. Adaptations to sulfur limitation include the expression of enzymes for scavenging sulfate [61, 131], expression of proteins with reduced sulfur content [8, 106], and expression of enzymes used for the transport and metabolism of alternate sulfur sources [159].

Sulfur limitation has been successfully applied to the enrichment of microorganisms active towards a variety of organosulfur compounds. Several examples are given in Table 2.1. Enrichment using sulfur limitation poses a challenge, due to the relatively low sulfur requirements for bacterial growth, as well as the repressive effect of sulfate on the use of organic sulfur sources by bacteria [75]. All but one of the studies listed in Table 2.1 mention special procedures used to minimize the effect of trace contaminant sulfur compounds. These procedures range from the use of specially cleaned glassware [175], to mutagenesis of the culture to increase desulfurization activity [77].

Table 2.1. Selected organic sulfur compounds successfully used for enrichment of microorganisms able to use the compounds as sole sulfur source under sulfur-limited conditions

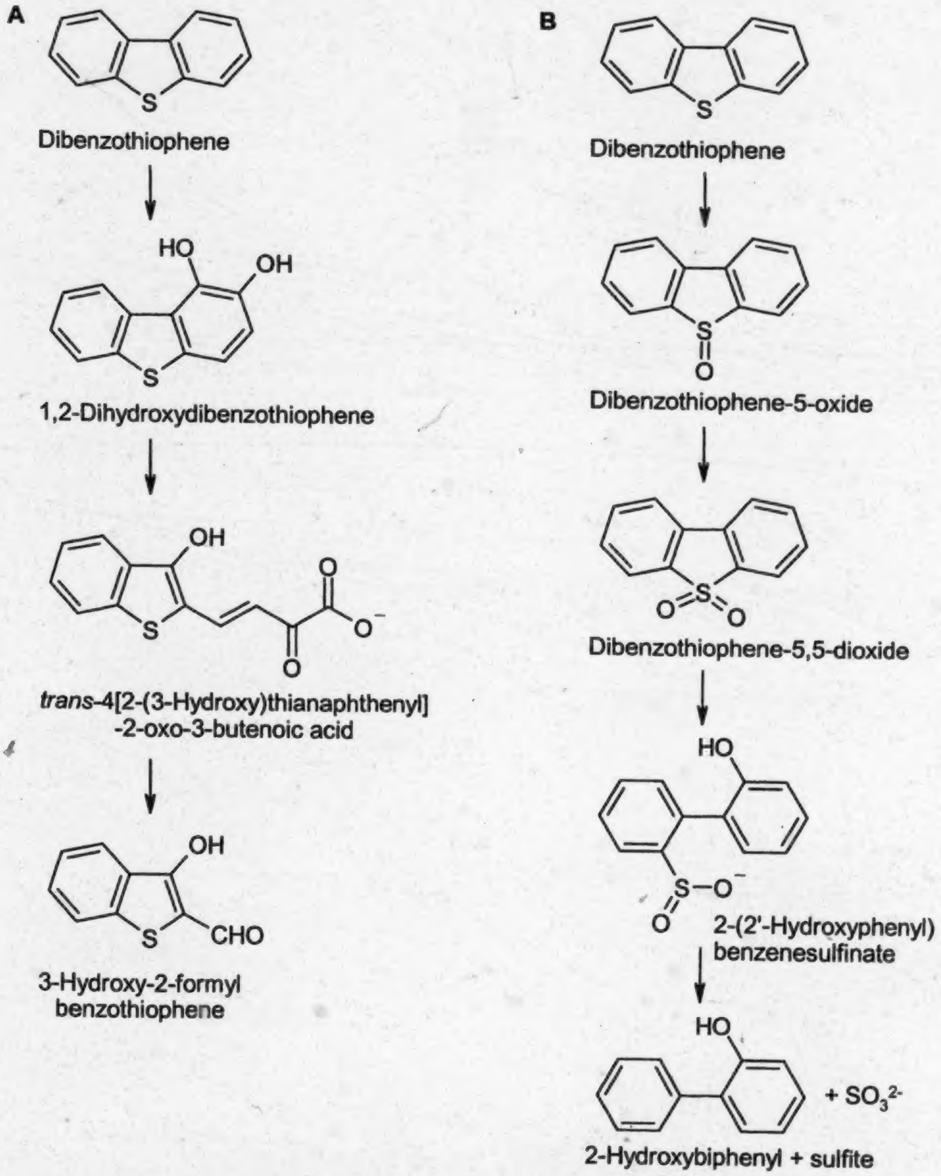
Compound	Procedures used	References
Ametryne and prometryne (herbicides)	Culture maintenance alternated between selective liquid and non-selective solid media	[29]
Naphthalenesulfonic acids Benzenesulfonic acids (detergents)	Substrate purification by high performance liquid chromatography “Scrupulously clean glassware” (procedure not given)	[175]
Organic sulfur in coal	Effluent from reactor mutagenized and reinoculated to accelerate strain evolution	[77]
Dibenzothiophene	(no special procedures given)	[120]
Endosulfan (an insecticide)	Use of an *Escherichia coli* culture to scavenge sulfate, followed by filter sterilization, to produce a sulfur-free medium	[154]

2.2.2 Biodesulfurization of thiophenic compounds

High-sulfur oils must undergo desulfurization during processing in order to meet emissions standards when burned. The possibility of biodesulfurization, using a biological catalyst to remove the sulfur, has been studied for decades. Aliphatic sulfides have not been targeted in oil desulfurization research. The relatively low carbon-sulfur bond strength results in easy cleavage of these bonds under thermal treatment compared to thiophenic sulfur, which is only removed during catalytic hydrotreating [51]. Correspondingly, research on biological desulfurization of oil has focused on the recalcitrant thiophenic compounds rather than the aliphatic sulfides, mainly using dibenzothiophene as a model for the sulfur compounds resistant to hydrodesulfurization.

Much of the early work looked at complete removal of dibenzothiophene, rather than selective sulfur removal (reviewed in [11]). Dibenzothiophene can be used as a carbon source for growth following the Kodama pathway [87], shown in Figure 2.3A. Dioxygenation of one aromatic carbon ring is followed by ring cleavage and chain degradation to produce 3-hydroxy-2-formyl benzothiophene.

A significant breakthrough in biodesulfurization research was the discovery of organisms that can selectively remove the sulfur from dibenzothiophene, leaving the carbon structure intact. This type of reaction preserves the fuel value of the substrate. The most extensively studied bacterium with the ability to desulfurize dibenzothiophene is *R. erythropolis* strain IGTS8 (ATCC 53968) (initially identified as *Rhodococcus rhodochrous* [79]). IGTS8 was isolated from a mixed enrichment culture active towards the organic sulfur in coal (Table 2.1). The mixed culture was also able to utilize dibenzothiophene as the sole sulfur source for growth, with the principal metabolites being dibenzothiophene-5-oxide (dibenzothiophene sulfoxide) and 2,2′-dihydroxybiphenyl [77]. Seven bacterial strains were isolated from the mixed culture, but only two showed desulfurizing activity towards dibenzothiophene: IGTS8; and isolate IGTS9, identified as *Bacillus sphaericus* (ATCC 53969) [79]. The only metabolite found in significant quantities was 2-hydroxybiphenyl for both microorganisms.



A
S
Dibenzothiophene
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OH
S
1,2-Dihydroxydibenzothiophene
OH
O
S
O⁻
O
trans-4[2-(3-Hydroxy)thianaphthenyl]
-2-oxo-3-butenoic acid
OH
S
CHO
3-Hydroxy-2-formyl
benzothiophene
B
S
Dibenzothiophene
S
O
Dibenzothiophene-5-oxide
O
S
O
Dibenzothiophene-5,5-dioxide
HO
S
O⁻
O
2-(2'-Hydroxyphenyl)
benzenesulfinate
HO
+ SO_3^{2-}
2-Hydroxybiphenyl + sulfite

Figure 2.3. **Dibenzothiophene biodegradation pathways: (A) carbon specific Kodama pathway (abbreviated) [87]; (B) sulfur specific 4S pathway of *R. erythropolis* strain IGTS8 [49].**

The pathway identified for desulfurization of dibenzothiophene by IGTS8, called the "4S" pathway, is shown in Figure 2.3B [49]. A two-step oxidation of the sulfur to dibenzothiophene-5,5-dioxide (dibenzothiophene sulfone) is followed by cleavage of one carbon-sulfur bond, giving 2-(2′-hydroxyphenyl)benzenesulfinate, and cleavage of the second carbon-sulfur bond releasing the sulfur as sulfite and leaving the carbon structure as 2-hydroxybiphenyl. Sulfite is oxidized abiotically to sulfate, which can be used as a sulfur source by the bacterium. IGTS8 can also use the aromatic sulfur heterocycle thianthrene as a sulfur source for growth, as well as a variety of other organic sulfur compounds including thiacycloalkanes (1,4,7-trithiacyclononane, 1,4-dithiane, and trithiane), and amino acids (methionine and cysteine) [71], but not the two-ring thiophenic compound benzothiophene [71] or the *n*-alkyl sulfide octyl sulfide [4].

Since the discovery of *R. erythropolis* IGTS8, several other bacteria have been isolated with the ability to desulfurize dibenzothiophene, a selection of which is given in Table 2.2. Other organic sulfur sources used by these organisms are also listed, to indicate their potential substrate range. Most of the bacteria studied are aerobic, mesophilic strains, but some aerobic thermophilic strains and anaerobic strains have also been found. The aerobic strains appear to use the same pathway, leading to 2-hydroxybiphenyl. Anaerobic bacteria studied are sulfate-reducing bacteria, which appear to use dibenzothiophene as a terminal electron acceptor, with the end products being the reduced compounds biphenyl and hydrogen sulfide.

Table 2.2. Selected dibenzothiophene-desulfurizing bacteria and alternate sulfur sources

Organism	Selected alternate organic sulfur sources used *	References
Aerobic mesophiles		
Rhodococcus sp. strain SY1 †	Dimethyl sulfide; benzene, p-toluene, 1-naphthalene, and 2-naphthalene sulfonate; methane and ethane sulfonate	[120, 121]
R. erythropolis strain D-1	Dimethyl sulfone; methanesulfonic acid; 2-mercaptoethanol	[65]
Gordonia sp. strain CYKS1	Dimethyl and benzyl sulfide; thiophene; 2-methyl thiophene; benzyl disulfide; trithiane	[133]
Nocardia sp. strain CYKS2	Dimethyl, benzyl, and phenyl sulfide; dimethyl, benzyl, and *p*-tolyl disulfide; thiophene; 2- and 3-methyl thiophene; benzothiophene	[23]
R. erythropolis strain KA2-5-1	4-Methyl DBT; 4,6- and 2,8-dimethyl DBT; 3,4,6-trimethyl DBT; 3,4,6,7-tetramethyl DBT; 2- and 3-ethyl DBT; 3-methyl, 7-ethyl, and 2,7-diethylbenzothiophenes	[86]
Aerobic thermophiles (up to 60°C)		
Paenibacillus sp. strain A11-2	2,8- and 4,6-dimethyl DBT; benzothiophene	[90, 92]
Mycobacterium phlei strain WU-F1	2,8- and 4,6-dimethyl DBT; 3,4-benzo DBT	[43]
M. phlei strain GTIS10	2-Methyl DBT; 4,8-dimethyl DBT; thiophene; 2-ethyl thiophene; thianthrene; trithiane; 1,3- and 1,4-dithiane; thiodiglycol; benzyl disulfide; dimethyl sulfoxide; benzothiophene	[72]
Anaerobes (sulfate-reducing bacteria)		
Desulfovibrio desulfuricans strain M6	Benzothiophene; phenyl and benzyl sulfide	[81]

* DBT, dibenzothiophene

† Originally identified as *Corynebacterium* sp. strain SY1 [120]

The genetic and enzymatic basis for the desulfurizing ability of *R. erythropolis* strain IGTS8 has been well characterized. A 4.0-kb DNA fragment was identified that conferred the full dibenzothiophene desulfurizing pathway [32]. This fragment was sequenced by two different research groups, and was found to encode three genes labeled *soxABC*, for sulfur oxidation [31], or *dszABC*, for desulfurization [126]. (The *dsz* designation appears to have been adopted in most of the desulfurization literature and will be used here. The *ABC* designations follow the order of the genes in the DNA sequence, and so are equivalent for the *sox* and *dsz* systems.) The enzymes encoded by these genes were identified as two monooxygenases and a desulfinase [49, 97], and the reactions catalysed are given in Table 2.3. A fourth enzyme was found to be required for proper expression of the desulfurization pathway. The monooxygenases require reduced nicotinamide adenine dinucleotide (NADH) and flavin mononucleotide (FMN) as cofactors for activity, but are unable to oxidize NADH. A NADH:FMN oxidoreductase, labeled DszD, was identified that performs this function [49].

Table 2.3. Enzymes identified in the desulfurization pathway of *R. erythropolis* strain IGTS8

Gene	Enzyme *	Reaction(s) catalysed *	References
dszC	DBT monooxygenase	DBT → DBTO DBTO → $DBTO_2$	[32, 97, 126]
dszA	$DBTO_2$ monooxygenase	$DBTO_2$ → HPBS	[32, 49]
dszB	HPBS desulfinase	HPBS → HBP + sulfite	[32, 49]
dszD	NADH:FMN oxidoreductase	FMN dependent NADH oxidation	[49]

* DBT, dibenzothiophene; DBTO, DBT-5-oxide; $DBTO_2$, DBT-5,5-dioxide; HPBS, 2-(2′-hydroxyphenyl)benzenesulfinate; HBP, 2-hydroxybiphenyl; NADH, reduced nicotinamide adenine dinucleotide; FMN, flavin mononucleotide

Only two reports were found of bacterial growth on the two-ring thiophenic compound benzothiophene as the sole carbon source [140, 169]. Growth of *Nocardioides* sp. strain PKSP 12 [140] and of *Mycobacterium* sp. strain SWU-4 [169] was assessed by turbidity only, with no confirmation of loss of the starting compound. Co-oxidation of benzothiophene by pure cultures has, on the other hand, been known for over 30 years [12, 17, 35, 40, 54, 108, 139]. Dioxygenation can occur on either ring, producing 2,3-

and 4,5-dihydrodiols [35], 2,3- and 4,5-diols [17, 35], and the 2,3-dione [35, 40]. Methyl- and dimethylbenzothiophenes are also cometabolized, generally yielding 2,3-diones if the substitution is on the hydrocarbon ring, and sulfoxides and sulfones if the substitution is on the thiophene ring [40, 93, 95, 138].

Like dibenzothiophene, benzothiophene can be used as a sulfur source to support growth. Use of benzothiophene as a sulfur source was first reported in *Acinetobacter* sp. strain EP [101], but the first well-characterized strain with this ability was *Gordonia* sp. strain 213E [47] (proposed as a new species, *Gordonia desulfuricans* strain 213E^T [82]), which does not use dibenzothiophene as a sulfur source. The ability to desulfurize benzothiophene has since been identified in several different organisms, a selection of which is given in Table 2.4. As indicated, some of these organisms can also use certain alkylated benzothiophenes and other organosulfur compounds as sulfur sources. Some dibenzothiophene-desulfurizing bacteria can also use benzothiophene (Table 2.2).

Two divergent pathways for the biodesulfurization of benzothiophene have been reported (Figure 2.4). As with the desulfurization of dibenzothiophene by *R. erythropolis* strain IGTS8 (Figure 2.3B, [49]), the initial reactions are monooxygenations at the sulfur atom, followed by carbon-sulfur bond cleavage reactions. Two different end products have been identified. In *G. desulfuricans* strain 213E, the sulfinate group is removed with oxygenation of the molecule, giving 2-(2′-hydroxyphenyl)ethan-1-al [47]. This product is recovered as benzofuran due to dehydration under acidic extraction conditions. In *Paenibacillus* sp. strain A11-2, the final product is *o*-hydroxystyrene, produced through desulfination of the molecule which does not oxygenate the carbon atom [92]. Only one organism, *Rhodococcus* sp. strain WU-K2R, has been reported to produce both end products from the desulfurization of benzothiophene [83]. WU-K2R also produces both analogous end products from the desulfurization of naphtho[2,1-*b*]thiophene [83].

Table 2.4. Selected benzothiophene-desulfurizing bacteria and alternate sulfur sources

Organism	Selected alternate organic sulfur sources used *	References
Paenibacillus sp. strain A11-2	Dibenzothiophene Cell-free extracts convert: 2-, 3-, 5-, and 7-methyl BT; 2,7- and 5,7-dimethyl BT; 2- and 7-ethyl BT; 7-propyl BT; 7-hexyl BT	[92] [91]
Rhodococcus sp. strain WU-K2R	3- and 5-methyl BT; naphtho[2,1-*b*]thiophene	[83]
Rhodococcus sp. strain T09	2-, 3-, and 5-methyl BT; 2-ethyl BT; benzene sulfonate and sulfinate; biphenyl sulfinate; dimethyl sulfide, sulfone, and sulfate; methane sulfonic acid; thiophene	[103]
Sinorhizobium sp. strain KT55	5-Methyl BT; dimethyl sulfide, sulfoxide, and sulfone; methanesulfonate	[157]
Gordonia rubropertinctus strain T08	2- and 5-methyl BT; 2-ethyl BT; benzene sulfonate and sulfinate; biphenyl sulfinate; methane sulfonate; dimethyl sulfone	[102]
Rhodococcus sp. strain KT462	2-, 3-, 5-, and 7-methyl BT	[156]
Mycobacterium goodii strain X7B †	3- and 5-methyl BT; dibenzothiophene; 4-methyl and 4,6-dimethyl dibenzothiophene; dimethyl sulfoxide; 3,3′-thiodipropionic acid; propyl mercaptan	[98]
Gordonia alkanivorans strain 1B †	Dibenzothiophene; 2-methyl thiophene; 2-mercaptoethanol	[2]

* BT, benzothiophene

† Isolates originally enriched on dibenzothiophene

Desulfurization of thiophenic species is not specifically of use for viscosity reduction in heavy crude oils. Removal of aromatic sulfur does not cleave the hydrocarbon backbone of the molecule, and therefore would not significantly reduce the molecular size of asphaltene molecules. The very specific sulfur removal mechanisms identified in organisms desulfurizing dibenzothiophene and benzothiophene do however provide an excellent model for the types of reactions required to biocatalytically cleave aliphatic sulfur bridges without reducing the fuel value of the material. The substrate ranges of these organisms for organic sulfur sources frequently include some alkylated species, and sometimes extend to compounds with aliphatic sulfide bonds as well, indicating that the ability to cleave aliphatic sulfur bridges may be closely related to the better-known biodesulfurization pathways for thiophenic compounds.

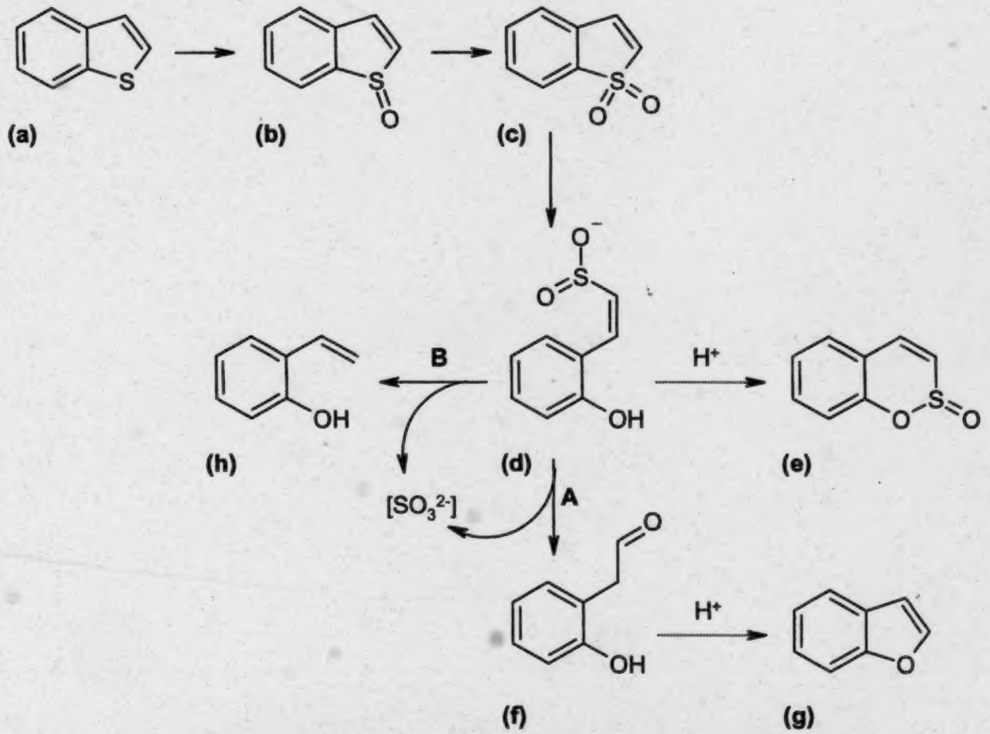


Figure 2.4. **Divergent pathways of benzothiophene desulfurization by (A) *G. desulfuricans* strain 213E [47], and (B) *Paenibacillus* sp. strain A11-2 [92]: (a) benzothiophene; (b) benzothiophene *S*-oxide (sulfoxide); (c) benzothiophene *S,S*-dioxide (sulfone); (d) 2-(2′-hydroxyphenyl)ethen 1-sulfinate; (e) benzo[*e*][1,2]oxathiin *S*-oxide (sultine); (f) 2-(2′-hydroxyphenyl)ethan-1-al; (g) benzofuran; (h) *o*-hydroxystyrene. Dotted arrows indicate abiotic reactions occurring under acidic extraction conditions.**

2.2.3 Biological oxidation of complex organic sulfur sources

Most studies of the biodegradation of organic sulfur sources have focused on pure compounds, either the substrate of interest (such as insecticides and herbicides), or a model compound representing a complex or mixed substrate (such as specific sulfonates to represent detergents, or dibenzothiophene to represent the sulfur in oils). A few studies have been done on the bacterial degradation of complex substrates containing organic sulfur. *R. erythropolis* strain IGTS8, for example, was originally isolated from an enrichment culture with the ability to use coal as its sole source of sulfur. The mixed culture was able to remove over 90 % of the organic sulfur from coal in a continuous flow reactor [78]. Organic sulfur would have been covalently bound within the coal matrix, primarily in thiophenic structures.

Fedorak and Coy [39] tested the ability of aerobic, heterotrophic bacterial cultures to utilize two different oil sands coke samples as sulfur sources, with acetate as a readily utilized carbon source. The coke samples were pretreated by Soxhlet extraction with water to remove any soluble sulfur species. In all their cultures containing Suncor coke and either *R. erythropolis* strain IGTS8 or *Bacillus* sp. strain IGTS9, sulfate accumulated in the medium concurrently with acetate consumption. Sulfate was not produced in coke-free controls, and therefore could only have been formed through release of sulfur from the coke matrix due to microbial activity.

The matrix of vulcanized rubber consists of carbon chains crosslinked by sulfide, disulfide, and polysulfide linkages. Bacterial attack appears to be limited to sulfur exposed at the surface of solid rubber particles, which is oxidized to sulfoxides and sulfones, and eventually released as sulfate [60].

The alkane-degrading bacterium *R. erythropolis* ATCC 13260 (originally reported as *Nocardioides simplex*) is able to degrade a high molecular weight fraction of crude oil [66]. This fraction contains 14.7 % sulfur, and was known from prior work to have hydrocarbon subunits linked by sulfide bridges. *R. erythropolis* ATCC 13260 degraded sulfur-bound linear alkanes and steranes in the oil fraction, leaving oxidized sulfur-bound species such as carboxylic acids. Sulfur-specific oxidation to sulfones was also observed, but no carbon-sulfur bond cleavage or desulfurization was reported [66].

In some reports, treatment of heavy crude oils with thermophilic bacteria led to an apparent enrichment in the lighter fractions of the oil [128-130]. This shift in composition was attributed to depolymerization of the asphaltene fraction of the oils, which was defined as the dissociation of small molecules either physically associated with or weakly chemically bound to asphaltenes. No significant quantitative change in the asphaltene content was measured. In addition, uniform removal of the range of sulfur compounds present in the oil was reported, which is not consistent with known chemical or biological conversion processes. Problems with sample recovery could account for some of the observed changes in oil composition; however, in the absence of appropriate controls allowing complete material or sulfur balances, definite conclusions cannot be drawn from this work.

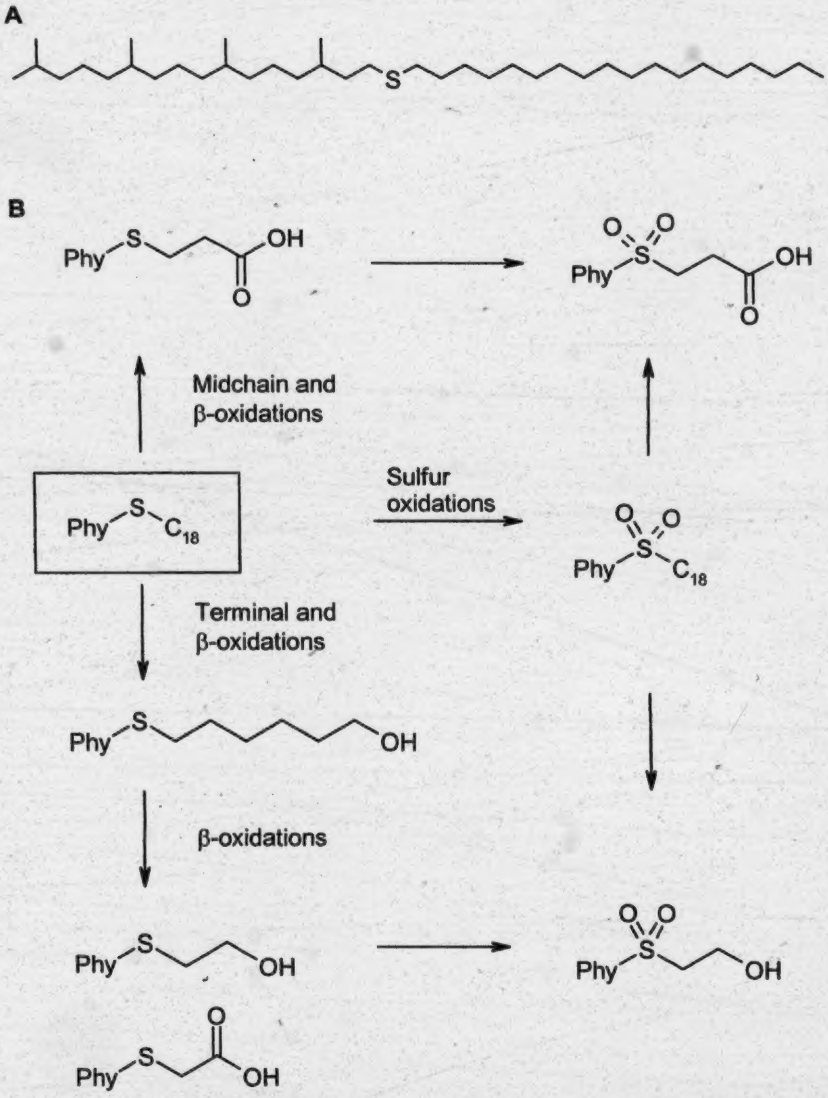
2.3 Biodegradation of aliphatic sulfides

The biodegradation of some aliphatic sulfides not related to oil has been studied, including dimethyl sulfide and analogues of sulfur mustard (2,2′-dichlorodiethyl sulfide). Biodegradation of some larger sulfides has been reported as well. These studies are presented here to illustrate the types of biological activity possible with aliphatic sulfides, and to show how they may be relevant to oil upgrading.

2.3.1 High molecular weight sulfides

Phytanyl octadecyl sulfide was used as a model compound for sulfur-bound hydrocarbons found in heavy oil macromolecules [66]. Biodegradation as a carbon source by *R. erythropolis* ATCC 13260 occurred only on the linear octadecyl chain, and not the branched phytanyl chain. In addition to the sulfone, six chain degradation metabolites were identified (Figure 2.5), which suggested two different mechanisms [66]. Metabolites with an even number of carbon atoms in the linear side chain were proposed by the authors to result from terminal oxidation followed by β-oxidations removing two carbon atoms at a time. Metabolites with an odd number of carbon atoms cannot arise solely from β-oxidations, and an initial mid-chain oxidation was proposed to occur as well. The appearance of primary alcohols as intermediates of chain degradation does not actually support the hypothesis of terminal oxidation followed by β-oxidations, since β-oxidation generates carboxylic acids as chain cleavage products [6]. Therefore, mid-chain cleavage reactions other than β-oxidation were also responsible for some of the metabolites with an even number of carbon atoms. Oxidation of the sulfur atom to a sulfone was observed both in the parent compound and in the degraded metabolites, indicating that sulfur oxidation was independent of the chain degradation pathways. No evidence of carbon-sulfur bond cleavage was reported [66].

Van Hamme *et al.* [162] showed fungal oxidation of the sulfur atom in benzyl sulfide, but again carbon-sulfur bond cleavage was not observed in this compound.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OH

Figure 2.5. **(A) Structure of phytanyl octadecyl sulfide. (B) Metabolites produced by *R. erythropolis* ATCC 13260 and reactions in the degradation of phytanyl octadecyl sulfide, as proposed by Jenisch-Anton *et al.* [66]. These reactions are discussed further in the text.**

Bacterial use of a high molecular weight aliphatic sulfide as a sulfur source has only recently been reported. A mutant of the dibenzothiophene-desulfurizing bacterium *R. erythropolis* strain IGTS8 is able to use *n*-octyl sulfide as a sulfur source, but this compound is not a substrate for the wild-type organism [4]. *Rhodococcus* sp. strain JVH1 is capable of using the novel compound *bis*-(3-pentafluorophenylpropyl) sulfide (PFPS) as its sole sulfur source for growth [160]. PFPS was specifically designed using ring fluorination to block any ring oxidation of the molecule, necessitating a subterminal attack to support growth. The desulfurization pathway proposed is shown in Figure 2.6. PFPS is first oxidized to the corresponding sulfoxide and sulfone (PFPSO and $PFPSO_2$). Carbon-sulfur bond cleavage then yields the primary alcohol 3-pentafluorophenylpropan-1-ol (PFPP-OH), which is further oxidized to 3-pentafluorophenylpropanoic acid (PFPP-acid). The second product of $PFPSO_2$ cleavage was proposed to be a sulfinate, analogous to the 4S pathway for dibenzothiophene desulfurization, but was not directly observed. Release of the sulfur as sulfite was also hypothesized but not directly observed. JVH1 was shown to use a variety of compounds with aliphatic carbon-sulfur bonds as sulfur sources (including dialkyl sulfides, thiacycloalkanes, and aryl-terminated sulfides), but not thiophenic compounds. This selective ability to cleave compounds with aliphatic carbon-sulfur bonds is extremely interesting for research into biological viscosity reduction in heavy crude oils.

For high molecular weight aliphatic sulfides, the mechanism of attack appears to depend on the substituent groups. Sulfur-bound alkyl chains are subject to aerobic degradation, apparently through the same pathways as *n*-alkanes [66]. Sulfur oxidation occurred independently, but did not prevent chain degradation [66]. A 4S-like pathway has been reported for the fluorinated compound PFPS. Interestingly, dibenzothiophene-desulfurizing strains only produced $PFPSO_2$, being apparently unable to cleave the aliphatic carbon-sulfur bonds in PFPS [160]. This result illustrated that sulfur-specific desulfurization of aliphatic sulfides and thiophenes may occur through analogous mechanisms, but that the desulfurization systems are not necessarily interchangeable.

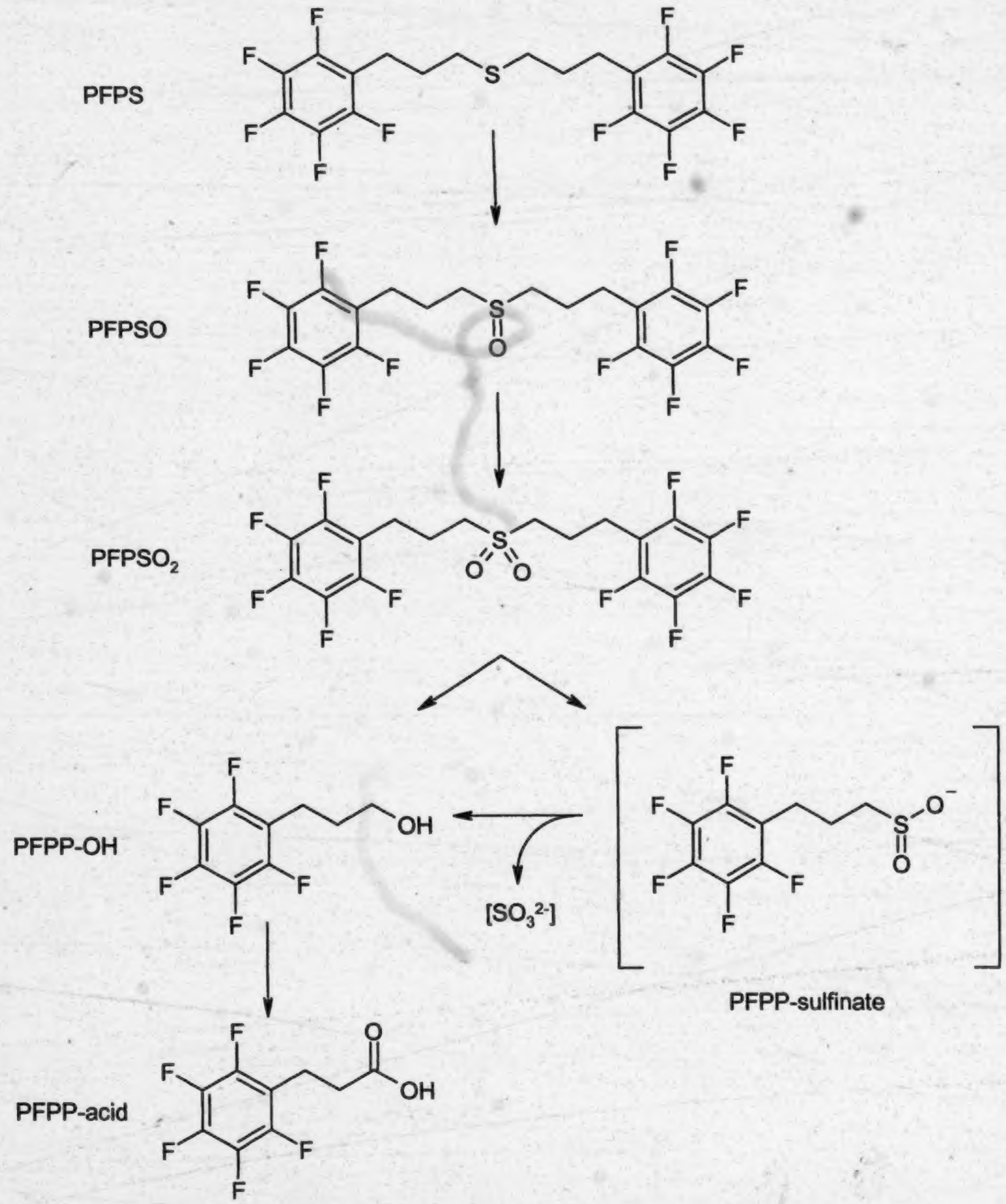
PFPS
F
S
PFPSO
O
PFPSO$_2$
PFPP-OH
OH
[SO$_3^{2-}$]
O$^-$
PFPP-sulfinate
PFPP-acid
OH

Figure 2.6. **Proposed pathway of PFPS metabolism in *Rhodococcus* sp. strain JVH1 [160] (PFPP-sulfinate, 3-pentafluorophenylpropane-1-sulfinate). Compounds in brackets were not directly observed.**

2.3.2 Dimethyl sulfide and related compounds

Dimethyl sulfide is part of the global sulfur cycle. It is formed in marine sediments from degradation of dimethylsulfoniopropionate, produced by marine algae and plants. Some dimethyl sulfide is released from the oceans to the atmosphere where it is involved in cloud formation, whereas most is degraded by a variety of marine microorganisms [44]. The pathway most studied, shown in Figure 2.7A, occurs under aerobic conditions in a variety of species of *Hyphomicrobium* and *Thiobacillus* [73]. Initial cleavage to methanethiol and formaldehyde is catalysed by a NADH-dependent monooxygenase. Methanethiol is then cleaved to a second molecule of formaldehyde and sulfide by methanethiol oxidase. Sulfide is further oxidized to sulfate. Formaldehyde is oxidized to formate, then to carbon dioxide by formaldehyde dehydrogenase and formate dehydrogenase.

Thiobacillus sp. strain ASN-1 can degrade dimethyl sulfide under both aerobic and anaerobic (nitrate-reducing) conditions [164]. Methanethiol, but not formaldehyde, was produced under aerobic conditions, suggesting a novel pathway for dimethyl sulfide degradation in this organism. The same degradative pathway was proposed for this organism under both aerobic and anaerobic conditions (Figure 2.7B), with the terminal electron acceptors being oxygen and nitrate, respectively. Each methyl group is removed by a methyltransferase and oxidized to formate. The sulfur is first released as sulfide, which is oxidized to sulfate. Sulfate was produced stoichiometrically under aerobic and anaerobic conditions. This work was extended to larger sulfides, and it was shown that ASN-1 could grow on diethyl sulfide, dipropyl sulfide, dibutyl sulfide, dimethyl disulfide, and dibutyl disulfide [163]. Growth on butanethiol, as well as on acetate, propionate, and butyrate, suggested that similar reaction mechanisms were used for the larger compounds as for dimethyl sulfide. Lag periods were observed when transferring cultures from one sulfide compound to another, but not from a sulfide to the corresponding thiol, indicating that different enzymes were used for the degradation of the different sulfides [163].

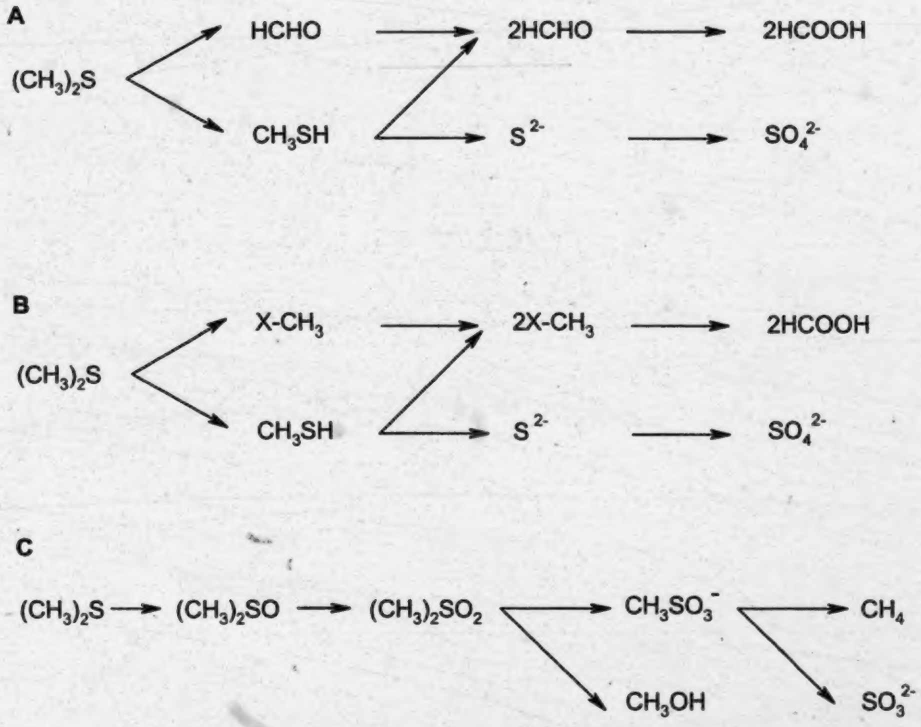
A
(CH3)2S
HCHO
CH3SH
2HCHO
S2-
2HCOOH
SO42-
B
(CH3)2S
X-CH3
CH3SH
2X-CH3
S2-
2HCOOH
SO42-
C
(CH3)2S
(CH3)2SO
(CH3)2SO2
CH3SO3-
CH3OH
CH4
SO32-

Figure 2.7. Dimethyl sulfide biodegradation pathways: (A) aerobic marine thiobacilli and hyphomicrobia [73]; (B) *Thiobacillus* sp. strain ASN-1 [164]; (C) *Rhodococcus* sp. strain SY1 [120]. (X – cobalamin carrier of methyltransferase)

Anaerobic degradation of dimethyl sulfide has been observed in marine sediments. Methanogenic consortia from these environments release methane from dimethyl sulfide, as well as from methanethiol and dimethyl disulfide [76]. Ethane release from the analogous compounds diethyl sulfide and ethanethiol has also been observed in marine sediment samples. Ethane release was not observed in killed controls, or in the presence of 2-bromoethanesulfonic acid, which inhibits methanogens [122].

The soil bacterium *Rhodococcus* sp. strain SY1, originally isolated for its ability to use dibenzothiophene as a sole sulfur source [120], can also degrade dimethyl sulfide as a sulfur source [121]. A pathway analogous to the sulfur-specific 4S pathway for dibenzothiophene was proposed (Figure 2.7C) based on the observed products from growth on successive intermediates. The sulfur atom in dimethyl sulfide is first oxidized giving dimethylsulfoxide and dimethyl sulfone. Growth on dimethylsulfoxide also showed release of dimethyl sulfone, as well as methanol and methane. Release of the sulfur as sulfite, which is spontaneously oxidized to sulfate under aerobic conditions, was proposed but never directly observed.

For the smallest aliphatic sulfide, dimethyl sulfide, degradation primarily occurs without oxidation of the sulfur atom, and has been observed under aerobic [73], nitrate-reducing [164], and methanogenic conditions [76]. Some larger sulfides may be subject to similar reactions, with butyl sulfide being the largest compound tested [163]. A 4S-like oxidative pathway has also been reported, allowing use of dimethyl sulfide as a sulfur source by a dibenzothiophene-desulfurizing organism [121]. This observation demonstrates that the dibenzothiophene-desulfurizing enzymes of some bacteria may also catalyse aliphatic carbon-sulfur bond cleavage.

2.3.3 Sulfur mustard and related compounds

Sulfur mustard (2,2′-dichlorodiethyl sulfide) was first used as a chemical warfare agent during World War I. The United States military is currently interested in degradation strategies for this compound for disposal of stockpiles at American military bases [63]. Similar research is also being done for disposal of Russian chemical weapons stockpiles [13]. Biodegradation of sulfur mustard is generally not studied directly because of its high toxicity. Proposed disposal processes start with chemical neutralization followed by treatments such as incineration, chemical oxidation, or biodegradation. In the neutralization step, sulfur mustard is hydrolysed to less toxic compounds, primarily the dechlorinated compound thiodiglycol [63], which has been the focus of most biodegradation studies. Chemical neutralization followed by biotreatment has been selected for disposal of mustard-containing chemical stockpiles at the U.S. Army Pueblo Chemical Depot in Colorado (Assembled Chemical Weapons Alternatives, U.S. Department of Defense, www.pmacwa.army.mil, accessed November 7, 2005).

Alcaligenes xylosoxydans ssp. *denitrificans* strains TD1 and TD2 are able to grow using thiodiglycol as the sole source of carbon and energy [37]. Strain TD2 is a variant of strain TD1, selected for a shorter lag period and higher specific growth rate on thiodiglycol. Identification of metabolites showed that both ends of the molecule were first oxidized to form thiodiglycol aldehyde and thiodiglycolic acid, followed by cleavage at the sulfur atom to form acetic acid and 2-mercaptoacetic acid. Up to 90 % of the sulfur finally accumulated in the medium as sulfate. Thiodiglycol sulfoxide was also formed, but did not support growth. The proposed pathways are shown in Figure 2.8A.

A. xylosoxydans ssp. *xylosoxydans* SH91 also uses thiodiglycol as its sole source of carbon and energy [96]. Thiodiglycol was oxidized via [(2-hydroxyethyl)thio] acetic acid to thiodiglycolic acid (Figure 2.8B) in two oxygen-dependent steps catalysed by butanol dehydrogenase. Thiodiglycol sulfoxide accumulated as a dead-end metabolite. Further metabolites were not shown analytically, but growth on thiodiglycol implied incorporation into cell mass and mineralization to CO_2 and sulfate.

A third strain of *A. xylosoxydans*, designated PGH10, is able to transform thiodiglycol in minimal medium with citrate or fructose [46]. Degradation was proposed to follow the same initial pathway as for *A. xylosoxydans* ssp. *xylosoxydans* SH91, based on observation of the same two initial metabolites, [(2-hydroxyethyl)thio] acetic acid and thiodiglycolic acid. Although PGH10 could transform thiodiglycol, there was no evidence for the release of carbon or sulfur from the metabolites. It appears that PGH10 may not be able to cleave the carbon-sulfur bond, resulting in its inability to use thiodiglycol as a carbon source.

Fungal degradation of sulfur mustard, thiodiglycol, and related compounds has also been studied. The white-rot fungus *Coriolus versicolor* (IFO 30340) degraded thiodiglycol most effectively in a high-carbon, low-nitrogen medium, and the brown-rot fungi *Tyromyces palustris* (strains IFO 0507 and IFO 30339) degraded thiodiglycol in sulfur-free medium [64, 168]. Both organisms were also able to degrade the brominated sulfur mustard analog 2,2′-dibromodiethyl sulfide, measured as loss of the starting compound [64]. Direct cleavage of the sulfide bond to form a mercaptan and an alcohol was observed for benzyl sulfide (forming benzyl mercaptan and benzyl alcohol) [64], thiodiglycol (forming 2-mercaptoethanol and ethylene glycol) [168], and sulfur mustard (forming 1-chloro-2-mercaptoethane and 2-chloroethanol) [168].

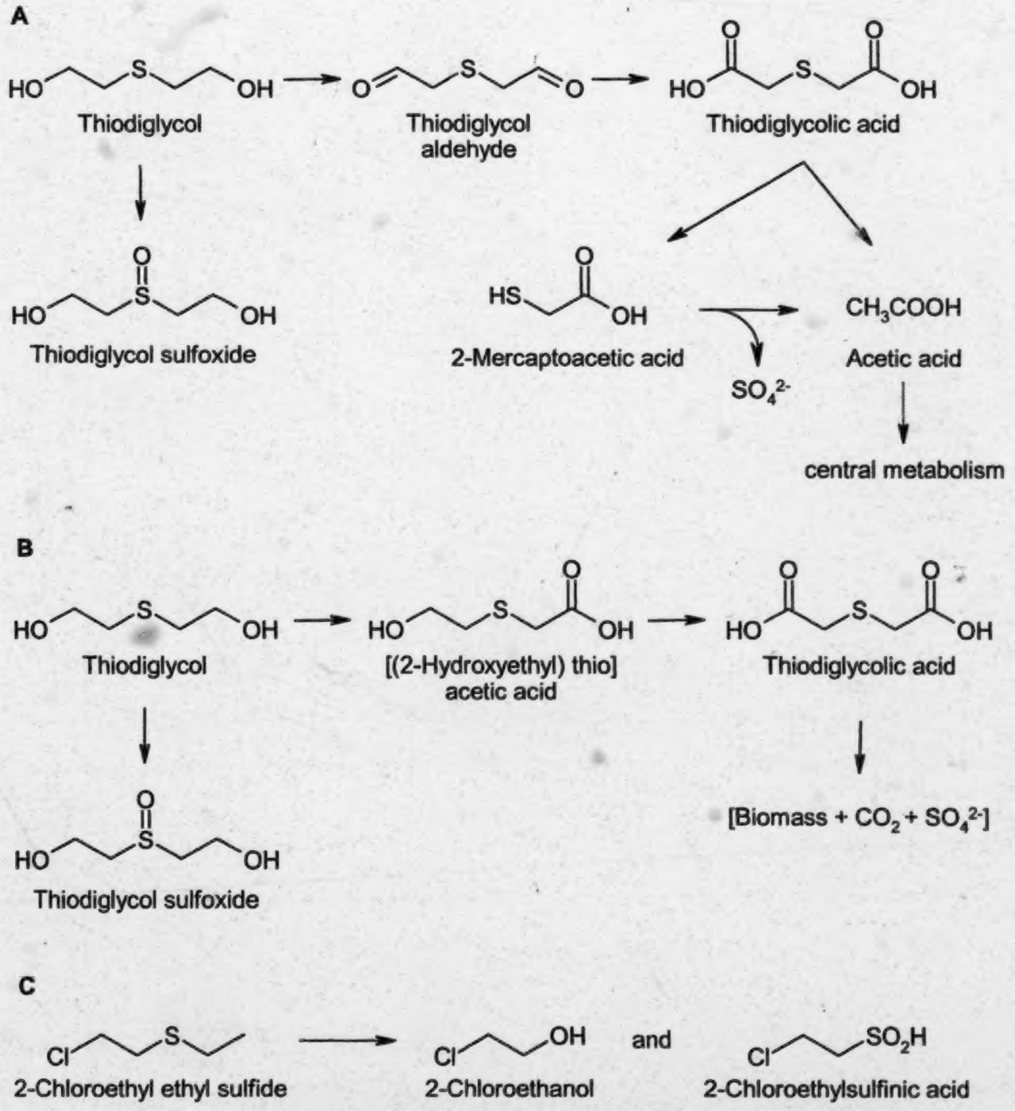
A
HO
OH
S
Thiodiglycol
O
O
Thiodiglycol aldehyde
HO
OH
Thiodiglycolic acid
Thiodiglycol sulfoxide
HS
2-Mercaptoacetic acid
SO_4^{2-}
CH_3COOH
Acetic acid
central metabolism
B
Thiodiglycol
[(2-Hydroxyethyl) thio] acetic acid
Thiodiglycolic acid
[Biomass + CO_2 + SO_4^{2-}]
Thiodiglycol sulfoxide
C
Cl
2-Chloroethyl ethyl sulfide
2-Chloroethanol
and
SO_2H
2-Chloroethylsulfinic acid

Figure 2.8. **Metabolites formed in the biodegradation of the sulfur mustard analogues (A) thiodiglycol by *A. xylosoxydans* ssp. *denitrificans* strain TD2 [37]; (B) thiodiglycol by *A. xylosoxydans* ssp. *xylosoxydans* strain SH91 [96]; and (C) 2-chloroethyl ethyl sulfide by *R. erythropolis* strain IGTS8 [80]. Products in brackets were not directly measured.**

One further study has investigated a sulfur-specific detoxification strategy for sulfur mustard. The dibenzothiophene-desulfurizing bacterium *R. erythropolis* strain IGTS8 can use the sulfur mustard analog 2-chloroethyl ethyl sulfide as a sole sulfur source for growth [80]. Two metabolites were found using gas chromatography and mass spectrometry (GC-MS). Growing cultures of *R. erythropolis* strain IGTS8 accumulated 2-chloroethylsulfinic acid, whereas 2-chloroethanol was found in resting cell cultures (Figure 2.8C). Neither compound was observed in killed cell controls.

For the sulfur-mustard analogue thiodiglycol, both sulfur oxidation and terminal carbon oxidation were observed in bacteria using the compound as a carbon source [37, 96]. These reactions were apparently independent and mutually exclusive with the sulfoxide produced accumulating as a dead-end metabolite. Carbon-sulfur bond cleavage was assumed to occur subsequent to terminal carbon oxidation, but only in the absence of sulfur oxidation. Some fungal strains were also able to degrade sulfur mustard and sulfur mustard analogues [64, 168]. As with dimethyl sulfide, 2-chloroethyl ethyl sulfide was subject to sulfur-specific degradation by a dibenzothiophene-desulfurizing strain [80], demonstrating again that the desulfurization enzymes may have a sufficiently broad substrate specificity to allow attack on both thiophenes and some aliphatic sulfides.

2.4 Solubility and mass transfer effects in biocatalysis of hydrophobic compounds

A biocatalytic treatment process for any oil feedstock will of necessity be a two-liquid-phase system, because the microbial cells require an aqueous environment, and the hydrocarbons have low water solubility and will form a separate organic liquid phase. Mass transfer from the organic phase to the cells is therefore a critical kinetic parameter for this type of system.

Bacterial cells are known to take up hydrophobic substrates by three mechanisms: cells can take up substrates dissolved the aqueous phase, they may take up substrates directly from the organic phase, or they may take up emulsified substrates when surfactants are present. Polycyclic aromatic compounds are more water soluble than saturated organic compounds of similar carbon number, and are generally taken up from the aqueous phase [143]. Medium and long chain length alkanes are much less soluble than aromatic compounds for a comparable size (number of carbon atoms). Alkane-degrading microorganisms may take up alkanes directly from the organic phase, or use surfactant-mediated uptake [15]. Direct uptake occurs at the organic-aqueous interface. Cells with this ability express a hydrophobic surface, allowing adhesion to the interface [14, 136]. Instead of obtaining hydrocarbons from the organic phase, many alkane-degrading microorganisms produce surfactants, resulting in "pseudo-solubilization", where small droplets of hydrocarbon are enclosed in micelles of surfactant molecules. The amphiphilic nature of the surfactant allows the micelles to be dispersed in the aqueous phase, making the hydrocarbon available for uptake by hydrophilic cells [9, 15].

Two transport processes are essential to the biotransformation of substrates dissolved in a second liquid phase: first, molecules must transfer from the organic phase to the cells; and second, molecules must be transported across the cell membrane to access enzymes inside the cells. Both processes can affect biotransformation rates through the rates of mass transfer and through the equilibrium partitioning of the substrate between the two phases. Bioavailability depends on the relative rates of mass transfer and of enzymatic conversion [33]. If the enzymatic reaction rate is relatively high compared to the overall mass transfer rate, biotransformation will be limited by the mass transfer rate and the substrate is said to have low bioavailability. Conversely, if the mass transfer rate is relatively high, biotransformation will be limited by the reaction rate and the substrate is said to have high bioavailability.

Partitioning has important effects on biotransformation in two-liquid-phase systems. The partition coefficient defines the relative concentrations of a substrate in two phases at equilibrium. For hydrophobic compounds, the organic concentration may be much higher than the aqueous concentration, which can affect biotransformation in two ways. First, partitioning may enhance biodegradation of toxic substrates by reducing the aqueous concentration below an inhibitory threshold [33]. Higher total substrate loadings can therefore be used and greater conversion can be achieved. This approach has been used for the biodegradation of compounds such as phenol and benzene (reviewed in [33]). Partitioning may also be detrimental to biotransformation if the aqueous concentration falls below a minimum threshold for enzymatic activity. The extent of conversion may then be reduced due to effective sequestering of the substrate in the organic phase [36].

Uptake by either contact mechanism can occur by passive diffusion, or by active (energy-dependent) transport into the cell [9]. The polarity of a compound, which affects its aqueous solubility and its organic-aqueous partitioning behaviour, influences the transport of the compound across the cell membrane by passive diffusion. Because the membrane is a lipid bilayer, the membrane interior is a hydrophobic environment where hydrophobic substrates may partition preferentially from the extracellular aqueous environment. Partitioning between organic and aqueous liquid phases is often represented by the octanol-water partition coefficient (K_{ow}). A quantitative correlation has been established between the oil-water partition coefficient and the membrane permeability, and Bressler and Gray [19] used this relationship to derive the following correlation between K_{ow} and the maximum flux ($F^{\max}$) across the cell membrane (equation 2.1):

$$F^{\max} = 0.003\frac{K_{ow}C_{aq}}{MW^{0.5}} \tag{2.1}$$

where C_{aq} is the saturated aqueous concentration and MW is the molecular weight of the compound. Therefore, the overall mass transfer rate used to determine bioavailability depends not only on the mass transfer of a compound from the organic phase to the cells in the aqueous phase, but also on the diffusion of the compound across the lipid membrane of the cells.

3. Materials and Methods

3.1 Chemicals

The sources and purities of purchased chemicals are given in Table 3.1. The commercial preparations of 1,4-dithiane and of benzyl sulfide were purified by recrystallization from methanol and methanol-water, respectively, for use in some experiments with isolates from enrichment cultures, and all experiments with *Rhodococcus* sp. strain JVH1.

The compounds PFPS, $PFPSO_2$, PFPP-OH, and PFPP-acid (all synthesized as described in [160]) were provided by Dr. J.D. Van Hamme of the National Centre for Upgrading Technology.

Benzothiophene sulfone and 3-methylbenzothiophene sulfone (synthesized as described in [20]), and 5-fluorobenzothiophene sulfone (synthesized as described in [18]) were provided by Dr. P.M. Fedorak from the Department of Biological Sciences, University of Alberta. Other alkylbenzothiophenes (synthesized as described in [3]) and alkylbenzothiophene sulfones (synthesized as described in [93]) were provided by Dr. P.M. Fedorak and Dr. J.T. Andersson from the Institute of Inorganic and Analytical Chemistry, University of Münster, Germany.

Table 3.1. Sources and purities of purchased chemicals

Source	Chemicals (purities)
Acros Organics (New Jersey)	*n*-Dodecyl sulfide (93%)
Aldrich (Milwaukee, WI)	Anthracene (99.9+%), benzothiophene (99%), benzyl disulfide (98%), benzyl sulfide (98%), benzyl sulfone (99%), carbazole (99%), cycloheptane (99%), cyclooctane (99+%), dibenzothiophene sulfone (99%), (±)-*trans*-3,4-dichlorotetrahydrothiophene 1,1-dioxide (99%) (also called 3,4-dichlorosulfolane), 2,4-dimethylsulfolane (95%), 1,4-dithiane (97%), 2-methylbenzofuran (96%), 2-methylbenzothiophene (97%), naphthalene (99+%), *n*-octyl sulfide (96%), phenanthrene (98%), phenyl sulfide (98%), 1-phenyldodecane (97%), tetrahydrothiophene (99%), tetramethylene sulfone (99%), tetramethylene sulfoxide (96%), thianthrene (99+%), and thiophene-2,5-dicarboxylic acid (99%)
Alfa Aesar (Ward Hill, MA)	5-Methylbenzothiophene (98%)
Caledon (Georgetown, ON)	Ethyl acetate (distilled in glass) and *n*-pentane (non-UV)
Eastman Kodak (Cleveland, OH)	*n*-Butyl sulfide
EM Science (Gibbstown, NJ)	Diethyl ether (anhydrous)
Fisher Scientific (Fairlawn, NJ)	Benzyl alcohol, dichloromethane (HPLC grade), methanol (HPLC grade), and 1-octanol
Fluka (Buchs, Switzerland)	Dibenzothiophene (≥98%) and benzyl sulfoxide (≥98.0%)
ICN (Plainview, NY)	*n*-Eicosane
Lancaster (Windham, NH)	3-Methylbenzothiophene
MC & B (Norwood, OH)	Benzoic acid
Pfaltz & Bauer (Stamford, CT)	*n*-Dodecylcyclohexane
Sigma (St. Louis, MO)	2,2,4,4,6,8,8-Heptamethylnonane (min. 95%), *n*-hexadecane (min. 99%), *n*-octane (99+%), and pristane (98%)
TCI America (Portland, OR)	*n*-Hexadecyl sulfide (98%), *n*-octadecyl sulfide (96+%)

3.2 Growth media, stock solutions, and amendments

Agar Noble, Bacto Agar, Plate Count Agar (PCA), and Trypticase Soy Broth (TSB) were from BD (Franklin Lakes, NJ). PCA and TSB were prepared using double distilled water. Half-strength PCA (½PCA) contained (per litre double distilled water): PCA (11.75 g) and Bacto Agar (7.5 g).

The formulations of the defined media and stock solutions used were as follows (DD H_2O, double distilled water; DI H_2O, deionized water):

Mineral media

B+NP Plates [41]

K_2HPO_4	0.5 g
NH_4Cl	1.0 g
Na_2SO_4	2.0 g
KNO_3	2.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$FeSO_4 \cdot 7H_2O$	trace
Agar Noble	15 g
DD H_2O	1.0 L

B+N8P [94]

Na_2SO_4	2.0 g
KNO_3	1.0 g
NH_4Cl	2.0 g
$FeSO_4 \cdot H_2O$	trace
DD H_2O	900 mL
Phosphate stock	100 mL
Trace metals solution	1.0 mL
$MgSO_4$ solution *	1.0 mL per 200 mL

* Added after autoclaving

Low Salts Basal

NH_4NO_3	28.6 g
KH_2PO_4	1.46 g
K_2HPO_4	1.87 g
K_2SO_4	0.54 g
DD H_2O	1.0 L

Adjusted to pH 7.2 with 10 N NaOH

Modified Bushnell-Haas (BHMV)

$MgSO_4$	0.2 g
$CaCl_2$	0.02 g
KH_2PO_4	1.0 g
K_2HPO_4	1.0 g
NH_4NO_3	1.0 g
$FeCl_3$	0.05 g
Trace metals solution	1.0 mL
DD H_2O	1.0 L
Pfennig's vitamins *	1.0 mL

* Added after autoclaving

Sulfur-free media

Sulfate-free mineral medium (SFMM)

K_2HPO_4	0.5 g
NH_4Cl	1.5 g
NaCl	1.0 g
$MgCl_2 \cdot 6H_2O$	0.1 g
Trace metals solution	1.0 mL
DI H_2O	1.0 L
Pfennig's vitamins *	1.0 mL

* Added after autoclaving

SFMM with acetate or glycerol (SFMM+2Ac, +4Ac, or +1% glycerol)
Prepared as SFMM with the addition of
2.0 g $CH_3COONa \cdot 3H_2O$ per litre (+2Ac),
4.0 g $CH_3COONa \cdot 3H_2O$ per litre (+4Ac),
or 10 g $C_3H_8O_3$ per litre (+1% glycerol)

Buffered SFMM+2Ac
Prepared as SFMM+2Ac, using
900 mL of DI H_2O and
100 mL of phosphate stock

Sulfate-free B+NP (S-free) [20]

K_2HPO_4	0.50 g
NaCl	0.82 g
$MgCl_2 \cdot 6H_2O$	0.33 g
NH_4Cl	1.0 g
KNO_3	2.0 g
$FeCl_2 \cdot 4H_2O$ *	trace
DI H_2O	1.0 L

* Added last once all ingredients dissolved

Adjusted pH to 7.3 with HCl or NaOH

S-free with acetate (S-free +2Ac)
Prepared as S-free with the addition of
2.0 g $CH_3COONa \cdot 3H_2O$ per litre

Stock solutions

Trace metals solution [40]

$CaCl_2 \cdot 2H_2O$	3.7 g
H_3BO_3	2.5 g
$FeCl_3$	0.65 g
$CoCl_2$	0.01 g
$ZnCl_2$	0.44 g
$MnCl_2$	0.87 g
$Na_2MoO_4 \cdot 2H_2O$	0.29 g
$CuCl_2$	0.0001 g
DD H_2O or DI H_2O •	1.0 L

• For mineral or sulfur-free media, respectively

Pfennig's vitamins [100]

p-Aminobenzoic acid	50 mg
Vitamin B-12	50 mg
Biotin	10 mg
Thiamine	100 mg
DD H_2O	1.0 L

Filter-sterilized (0.22 µm) and added to sterile, cooled medium

$MgSO_4$ solution

$MgSO_4 \cdot H_2O$	4.0 g
DD H_2O	100 mL

Autoclaved and added to sterile medium

Phosphate stock

KH_2PO_4	16.0 g
K_2HPO_4	24.0 g
DD H_2O or DI H_2O •	1.0 L

• For mineral or sulfur-free media, respectively

Phosphate buffer

In DD H_2O, prepared:

KH_2PO_4	20.3 g per 150 mL
K_2HPO_4	60.96 g per 350 mL

The KH_2PO_4 solution was added to the K_2HPO_4 solution until a pH of 7.2 was reached.
The buffer was diluted 100× in DD H_2O and sterilized before use (giving 10 mM).

All glassware used with sulfur-free media was washed with 4 M HCl, dichloromethane, and deionized water before use to remove trace amounts of inorganic and organic sulfur.

n-Octadecyl sulfide, *n*-octyl sulfide, and 1,4-dithiane were added directly to sterile medium for enrichment cultures. Sulfate was added as Na_2SO_4 before autoclaving or as an autoclaved solution of Na_2SO_4 in deionized water. L-Cysteine was added to sterile medium as a filter-sterilized (0.22 µm) solution in deionized water. *n*-Dodecyl sulfide and *n*-hexadecyl sulfide were added in *n*-pentane to tubes of sterile medium, and the solvent was allowed to evaporate before capping the tubes. For pure cultures, crystalline *n*-octadecyl sulfide was added to sterile glassware in dichloromethane, which was allowed to evaporate before adding sterile medium. Other sulfur sources were added in a minimum volume of methanol, or as filter-sterilized (0.22 µm) solutions in 2,2,4,4,6,8,8-heptamethylnonane (HMN).

For experiments using high salt concentrations, NaCl was added to the medium (TSB or SFMM+4Ac) before autoclaving. For experiments with sulfate analogues, selenate, chromate, molybdate, and tungstate were added to sterile medium (TSB or

SFMM+4Ac) as filter-sterilized (0.22 µm) solutions of their sodium salts in deionized water.

For alkane degradation experiments, *n*-hexadecane and HMN were filter-sterilized (0.22 µm) before adding to sterilized BHMV. Soluble carbon sources were added to sterile BHMV (1:10 dilution) as filter-sterilized (0.22 µm) solutions in deionized water, giving a final concentration of 0.01 mol of carbon per litre of medium. Soluble carbon sources used were glucose ($C_6H_{12}O_6$), fructose ($C_6H_{12}O_6$), mannose ($C_6H_{12}O_6$), ribose ($C_5H_{10}O_5$), sucrose ($C_{12}H_{22}O_{11}$), glycerol ($C_3H_8O_3$), mannitol ($C_6H_{14}O_6$), sodium acetate ($NaC_2H_3O_2$), propionic acid ($C_3H_6O_2$), sodium pyruvate ($NaC_3H_3O_3$), 4-hydroxybutyric acid ($C_4H_8O_3$), and sodium benzoate ($NaC_7H_5O_2$).

3.3 Enrichment cultures and isolation of potential microorganisms

3.3.1 Sources and maintenance of cultures

Mixed cultures for enrichment studies were obtained from two primary sources. Laboratory cultures (Table 3.2) were mixed cultures obtained from enrichments in previous work, which were being stored or maintained by the Department of Biological Sciences (Microbiology). These cultures had activity towards oil or towards sulfur-containing compounds. Environmental samples were water samples (Table 3.3) and soil samples (Table 3.4) collected from sites exposed to or contaminated with hydrocarbon-type materials. The laboratory cultures and the mixed cultures obtained from water samples were maintained in liquid media (200 mL S-free +2Ac medium or B+N8P medium in 500-mL Erlenmeyer flasks) with oil, bitumen, or a sulfur compound provided as the sole sulfur or carbon source. These cultures were incubated on rotary shakers at 200 rpm in the dark at 28°C (Husky, New Composite, SL vs. 83-02, Eco Pit, and Cold Lake cultures) or at room temperature (about 20°C). Soil samples were maintained in roller bottles [52] with a liquid medium (S-free +2Ac medium or Low Salts Basal medium), incubated on a bottle roller in the dark at room temperature (about 20°C). Heavily hydrocarbon-contaminated soils had no additional carbon or sulfur source, whereas less contaminated soils had bitumen added.

Table 3.2. Laboratory cultures used to start enrichment cultures

Name	Description	Maintenance substrate * (limiting element †)
Husky	Combined inoculum of Husky fungal dibenzothiophene-sulfoxide- and dibenzothiophene-sulfone-degrading mixed fungal and bacterial cultures	83-02 oil (S)
Minas	Maintenance culture degrading Minas heavy crude oil	Minas crude oil (S, C)
New Composite	Enrichment culture from Lloydminster oily sludge landfarming operation, originally enriched for desulfurizing activity	83-02 oil (S)
SL vs. 83-02	Shell Lake maintenance culture, maintained on 83-02 oil	83-02 oil (S, C)

* 83-02 oil – Lloydminster heavy crude oil
† S – sulfur source only (using S-free +2Ac medium); C – carbon source only (using B+N8P medium)

Table 3.3. Water samples used to start enrichment cultures

Name	Description	Maintenance substrate * (limiting element †)
K1	Contaminated groundwater from Norman Wells; showed growth on oil by most probable number counts	83-02 oil (S) 0464 bitumen (S)
K4	Contaminated groundwater from Norman Wells; showed growth on oil by most probable number counts	83-02 oil (S) 0464 bitumen (S) *n*-Octadecyl sulfide (S)
K6	Contaminated groundwater from Norman Wells; showed growth on oil by most probable number counts	83-02 oil (S)
K7	Contaminated groundwater from Norman Wells; showed growth on oil by most probable number counts	83-02 oil (S) 0464 bitumen (S)
Eco Pit ‡	Oily water from Eco-Pit of Cold Lake facility	UTF bitumen (S, C)
Cold Lake ‡	Produced water from Cold Lake facility	UTF bitumen (S)
Syncrude Mature Fine Tailings	Tailings pond sludge	*n*-Octyl sulfide (S) *n*-Octadecyl sulfide (S)

* 83-02 oil – Lloydminster heavy crude oil; 0464 bitumen – whole bitumen from Syncrude Canada; UTF bitumen – Athabasca bitumen from *in situ* production
† S – sulfur source only (using S-free +2Ac medium); C – carbon source only (using B+N8P medium)
‡ Sampled at Imperial Oil Ltd Cold Lake facilities, November 22, 1999

Table 3.4. Soil samples used to start enrichment cultures

Name	Description	Maintenance substrate * (limiting element †)
Devon ‡	Crude oil contaminated soil, Devon AB	0464 bitumen (C)
Edmonton ‡	Creosote contaminated soil, Edmonton AB	0464 bitumen (C)
Montreal ‡	Soil contaminated with refinery sludges, Montreal QC	0464 bitumen (C)
Prince Albert ‡	Creosote contaminated soil, Prince Albert SK	0464 bitumen (C)
Fort Mac #1	Weathered Athabasca oil sand, Hanging Stone River, Goodwin Pl., creek side slide	Hydrocarbons in soil (S, C)
Fort Mac #2	Athabasca oil sand, Hanging Stone River, Goodwin Pl., outcrop	Hydrocarbons in soil (S, C)
Fort Mac #3	Weathered Athabasca oil sand, Hanging Stone River, Goodwin Pl., creek side slide, creek level	Hydrocarbons in soil (S, C)
Pan Canadian	Core sample, 383.0 m	Hydrocarbons in soil (S, C)
Landfarm D-11 §	Soil from Cold Lake facility	UTF bitumen (S)
V Pad Battery §	Soil from Cold Lake facility	UTF bitumen (S)
Eco Pit Wellhead §	Oily sand from Cold Lake facility	UTF bitumen (S)
Pad B6 Manifold §	Soil from Cold Lake facility	UTF bitumen (S)
May Plant #1 soil §	Soil from decommissioned plant at Cold Lake	UTF bitumen (S)
May Plant #2 soil §	Soil from decommissioned plant at Cold Lake	UTF bitumen (S)

* 0464 bitumen – whole bitumen from Syncrude Canada; UTF bitumen – Athabasca bitumen from *in situ* production

† S – sulfur source only (using S-free +2Ac medium); C – carbon source only (using Low Salts Basal medium)

‡ For additional information on these petroleum and creosote contaminated soils, see ref. [137]

§ Sampled at specified locations at Imperial Oil Ltd Cold Lake facilities, November 22, 1999

3.3.2 Carbon-limited enrichment cultures

Enrichment cultures were attempted using the laboratory cultures and the environmental water samples with *n*-octyl sulfide, *n*-octadecyl sulfide, or 1,4-dithiane as the sole carbon and sulfur source for growth. A subsample of each maintenance culture (0.5 mL) was added to 5 mL S-free medium with 50 μL of *n*-octyl sulfide or 2.5 mg of *n*-octadecyl sulfide, or to 5 mL SFMM with 0.3 mg of 1,4-dithiane in screw-cap test tubes. The tubes were incubated on a tube roller in the dark at room temperature (about 20°C). If growth was evident, 0.5 mL of each culture was transferred weekly into fresh medium. Once growth was maintained for several transfers, isolates were purified for further screening.

3.3.3 Sulfur-limited enrichment cultures

Enrichment cultures were established using the laboratory cultures and the environmental water and soil cultures with *n*-octyl sulfide, *n*-octadecyl sulfide, or 1,4-dithiane as the sole sulfur source for growth. For the laboratory cultures and water cultures, a subsample of each maintenance culture (0.5 mL) was added to 5 mL SFMM+2Ac medium with 50 µL of *n*-octyl sulfide, 2.5 mg of *n*-octadecyl sulfide, or 0.3 mg of 1,4-dithiane in screw-cap test tubes. Tubes were incubated on a tube roller in the dark at room temperature (about 20°C). A subsample of each culture (0.5 mL) was transferred weekly into fresh medium. For the soil cultures, a subsample of each maintenance culture (approximately 10 mL) was added to 200 mL Buffered SFMM+2Ac with 0.025 g *n*-octadecyl sulfide in 500-mL Erlenmeyer flasks. Flasks were incubated on a rotary shaker at 200 rpm in the dark at room temperature (about 20°C). A subsample of each culture (10 mL) was transferred monthly into fresh medium. After several transfers, sulfur-limited enrichment cultures were screened to identify active cultures before purification of isolates for further testing.

3.3.4 Comparative growth assay for sulfur-limited cultures

Enrichment using sulfur limitation poses a technical challenge due to the relatively low sulfur requirements for bacterial growth. A comparative growth assay (CGA) was developed to identify interesting sulfur-limited enrichment cultures by comparing growth with a model compound to growth with no added sulfur (negative control) and growth with sulfate (positive control). The CGA was also used to screen bacterial isolates purified from the enrichment cultures.

Cultures were grown in 5 mL of SFMM+2Ac (negative control); SFMM+2Ac with *n*-octyl sulfide, *n*-octadecyl sulfide, or 1,4-dithiane (depending on which compound was used for enrichment); and SFMM+2Ac with sulfate (Na_2SO_4; positive control), in screw-cap test tubes. All sulfur compounds had a nominal concentration of 1 mmol L^{-1} except 1,4-dithiane (0.5 mmol L^{-1}, giving a sulfur concentration of 1 mmol L^{-1}). Tubes were incubated on a tube roller in the dark at room temperature (about 20°C). Growth was monitored by measuring optical density at 600 nm (OD_{600}) directly in the culture tubes (Section 3.6.1). If culture growth with the sulfur compound was greater than the negative control, the culture was assumed to be active towards that compound as a sulfur source.

3.3.5 Purification of isolates from enrichment cultures

Microbial isolates were purified from active enrichment cultures through dilution plating and repeated subculturing on ½PCA. Serial dilutions from 10^{-1} to 10^{-5} were prepared from the cultures in phosphate buffer. Plates of ½PCA were inoculated from the three highest dilutions by spreading 100 µL of the suspension over the agar surface with a glass spreader. The plates were stored in the dark at room temperature (about 20°C) until colony morphology could be distinguished (approximately 1 week). Isolated colonies were repeatedly picked and streaked on fresh plates until pure cultures were obtained (as determined by colony morphology).

3.4 Characterization and testing of isolates from enrichment cultures

3.4.1 Biochemical characterization and identification of selected isolates

The Gram reaction was assayed using the 3 % KOH test [165]. Other biochemical tests performed were the Gram stain [112], the acid-fast stain [112], and the catalase test [147].

Selected bacterial isolates were identified using the Biolog MicroPlate bacterial identification system (Biolog Inc., Hayward, CA). Isolate EPWF was also identified through sequencing of the full 16S rRNA gene (performed by MIDI Labs, Newark, DE) and comparison of the sequence to the GenBank database (BLAST search performed November 7, 2004, http://www.ncbi.nlm.nih.gov/BLAST, [1]). The 16S rRNA gene sequence for isolate EPWF was deposited to the GenBank database with accession number AY822047.

3.4.2 Use of hydrocarbons and aromatic heterocycles as carbon sources

Isolates were tested for the ability to use hydrocarbons and heterocyclic aromatic compounds as carbon sources using spray plates (adapted from the method of Kiyohara *et al.* [85]), spread plates, or vapour plates. Spray plates were used for non-volatile, solid substrates (*n*-eicosane, anthracene, phenanthrene, dibenzothiophene, and carbazole). An isolate was first streaked on a B+NP plate. The substrate was dissolved in a volatile solvent (*n*-pentane for *n*-eicosane, diethyl ether for the aromatic compounds) and sprayed over the agar surface using a Jet Pak Spray System (Sherwin-Williams Company, Bedford Heights, OH), to create a thin film of the substrate. Spread plates were prepared for non-volatile liquid substrates (*n*-hexadecane, pristane, *n*-dodecylcyclohexane, 1-phenyldodecane, HMN, 1-octanol) by spreading 10 µL of the substrate over the surface of a B+NP plate using a sterile glass spreader before inoculation. Vapour plates were used for volatile substrates (*n*-octane, cycloheptane, cyclooctane, naphthalene). Liquid substrates were added to glass tubes stuffed with glass wool. After streaking an isolate onto a B+NP plate, either a tube containing the substrate was attached inside the lower lid of the plate, or solid crystals (naphthalene) were placed in the lower lid of the plate. The plates were sealed in separate plastic bags to prevent cross-contamination with vapours and incubated in the dark at room temperature (about 20°C). Negative controls received no substrate. The ability to use a substrate as a carbon source was observed as clearing of the substrate on spray plates, or growth on spread plates and vapour plates (compared to the negative control).

To assess the toxicity of selected compounds, spray plates, spread plates, or vapour plates were prepared using PCA. Growth indicated that a compound was not toxic.

3.4.3 Bacterial adhesion to hydrocarbons assay

The relative hydrophobicity of isolates EPWF, K1oA, and K1bD was assessed using the bacterial adhesion to hydrocarbons (BATH) assay [135], with *Acinetobacter venetianus* strain RAG-1 as a hydrophobic positive control and *Pseudomonas fluorescens* strain LP6a as a hydrophilic negative control [34]. Cultures were grown overnight in TSB, harvested by centrifugation, and resuspended in phosphate buffer to an OD_{600} of less than 0.5. Various volumes of *n*-hexadecane were mixed with 1.2 mL of cell suspension using a vortex mixer at high speed for 120 s, and then allowed to settle for 15 min. The OD_{600} of the aqueous phase was measured again, and the percentage change in OD_{600} was calculated as an indicator of how many cells adhered to the organic phase.

3.4.4 Testing for biotransformation of organic sulfur compounds

For most experiments, isolates were grown in 5 mL of SFMM with various sulfur sources in culture tubes (Kimax brand 16×150 round-bottom tubes with screw-caps fitted with PTFE liners). The tubes were incubated on a tube roller in the dark at room temperature (about 20°C).

To test for biotransformation of *n*-octadecyl sulfide, EPWF was grown in 100 mL SFMM+4Ac containing 1 mg of substrate added in 1 mL of HMN in baffled 250-mL Erlenmeyer flasks. The flasks were incubated for 2 weeks on a rotary shaker at 200 rpm in the dark at room temperature (about 20°C).

To test for biotransformation of *n*-octyl sulfide, isolates EPWF, K1oA, and K1bD were grown in 200 mL of SFMM+4Ac, and isolate K7b1 was grown in 200 mL of SFMM containing sulfate (0.5 mmol L^{-1}), in 500-mL Erlenmeyer flasks. Each flask contained 5 mg of *n*-octyl sulfide added in methanol.

To identify acidic metabolites of *n*-octyl sulfide, isolate EPWF was grown in 200 mL of SFMM with glycerol (2 g L^{-1}), *n*-octyl sulfide (50 µL, added directly to the culture), and ion-exchange resin (Amberlite IRA-92, Supelco, Bellefonte, PA; 3.0 g per flask), in 500-mL Erlenmeyer flasks. The flasks were incubated for 3 days on a rotary shaker at 200 rpm in the dark at room temperature (about 20°C). After incubation, the resin was transferred to a glass chromatography column and the spent medium was collected. The resin was washed with 100 mL deionized H_2O, which was also collected and pooled with the spent medium. To recover anionic organic products from the resin, 250 mL of ion-exchange solvent [a solution of NaCl (0.5 mmol L^{-1}) and ethanol (50 %) in deionized water] was passed through the column and collected.

Sterile (uninoculated) controls were included for all substrates used in each experiment.

3.5 Experimental conditions with *Rhodococcus* sp. strain JVH1

Rhodococcus sp. strain JVH1 was stored in glycerol stocks at -70°C. To start an experiment, JVH1 was first streaked from frozen stocks onto PCA and grown for 3 to 14 days. Single colonies were picked from the PCA plate to inoculate B+NP plates to screen for use of hydrocarbons and heterocyclic aromatic compounds as outlined in Section 3.4.2. For experiments in liquid cultures, a single colony was picked from the plate to inoculate a suitable liquid medium (Table 3.5). After growth, a portion of the culture was harvested by centrifugation, washed three times and resuspended in an equal volume of phosphate buffer. Portions of the final suspension were used to inoculate experimental cultures. The media, growth times, and inoculation size used for the different experiments are outlined in Table 3.5.

Table 3.5. Inoculum conditions used for experiments with *Rhodococcus* sp. strain JVH1

Type of experiment	Medium used to grow inoculum	Size of inoculum cultures	Time to grow inoculum (days)	Size of inoculum used (relative to final culture volume)
Growth with organic sulfur sources	SFMM+4Ac with 0.025 mmol 1,4-dithiane L^{-1}	5 mL	3	0.4 %
Resting cells	SFMM+4Ac with 0.025 mmol 1,4-dithiane L^{-1}	100 mL	4	100 %
Salt tolerance	TSB	10 mL	1	0.05 % *
Sensitivity to sulfate analogues	TSB	10 mL	1	0.6 % *
Degradation of HMN	TSB	10 mL	1	0.1 % (live cultures) 1 % (killed controls)
Degradation of *n*-hexadecane	TSB	100 mL	2	10 %

* inoculum transferred directly without centrifugation or washing

Most liquid cultures were grown or incubated in 5 mL of SFMM+4Ac with an appropriate sulfur source in culture tubes (Kimax brand 16×150 round-bottom tubes with screw-caps fitted with PTFE liners). Cultures with *n*-octadecyl sulfide with and without HMN were grown in 100 mL of SFMM+4Ac in baffled 250-mL Erlenmeyer flasks.

Cultures with HMN with and without *n*-hexadecane were grown in 100 mL of BHMV in baffled 250-mL Erlenmeyer flasks. Other cultures with HMN were grown in 100 mL of SFMM+4Ac in 250-mL Erlenmeyer flasks with PTFE-lined screw caps to prevent evaporative losses. To test salt tolerance, cultures were grown in 100 mL of TSB with different added NaCl concentrations in 250-mL Erlenmeyer flasks.

Tubes were incubated on a tube roller, and flasks were incubated on a rotary shaker at around 160 rpm. All cultures (plates, tubes, and flasks) were incubated in the dark at 28°C.

3.6 Analytical methods

3.6.1 Growth measurement using optical density

Relative growth in culture tubes was monitored by measuring OD_{600} without dilution directly in the tubes with a Bausch & Lomb Spectronic 21 spectrophotometer, using a sterile control tube as a blank, and including sulfur-free negative controls for comparison. Growth data measured in this way are reported as "qualitative OD_{600}", and are only compared within an experiment for a single bacterial strain. Growth in TSB for the salt tolerance experiment was measured as the OD_{600} of subsamples with sterile TSB as a blank, using a Philips model PU 8740 UV/VIS Scanning Spectrophotometer. OD_{600} measurements for the BATH assay used the same instrument, with phosphate buffer as a blank.

3.6.2 Liquid-liquid extraction of cultures

After incubation, all cultures and other samples to be extracted were acidified with concentrated HCl to pH<1, and either extracted immediately or stored at 4°C until extraction.

For tube cultures, an internal standard was added (HMN for *n*-hexadecane cultures, benzothiophene for 1,4-dithiane cultures, 1,4-dithiane for benzothiophene cultures, 1-phenylnaphthalene for PFPS cultures, *n*-octyl sulfide for thianthrene cultures, thianthrene for all other cultures) and the cultures were extracted with 2 mL or 3 mL of dichloromethane (cultures with organic sulfur sources), or 5 mL of dichloromethane (cultures with *n*-hexadecane). After adding dichloromethane to the culture tube, the two phases were mixed using a vortex mixer at high speed. After phase separation, the organic layer was removed to a vial using a Pasteur pipette.

For flask cultures with organic sulfur sources (except the model oil experiments), thianthrene was added as an internal standard. For the flask experiment with HMN and *n*-hexadecane, *n*-dodecane was added as an internal standard. For screening of isolates from carbon-limited enrichment cultures, the cultures were extracted three times with 25 mL of ethyl acetate. Cultures with *n*-octadecyl sulfide in HMN were extracted three times with 50 mL of dichloromethane. Other cultures were extracted three times with 25 mL of dichloromethane using a separatory funnel. Water was removed from the organic phase by filtration through anhydrous sodium sulfate into a round bottom flask. The extract was concentrated to less than 5 mL at reduced pressure on a rotary evaporator, then transferred to a vial and dried completely under a nitrogen stream. The sample was then redissolved in a suitable volume of dichloromethane for gas chromatographic analysis.

For the model oil experiments (flask cultures with benzothiophene and benzyl sulfide added in HMN), 5 mL of *n*-pentane was added then *n*-hexadecane in a small volume of dichloromethane as an internal standard. The flasks were swirled vigorously and allowed to settle, then tap water was added at the bottom of the flask through a glass tube to raise the *n*-pentane layer into the neck of the flask. A sample of the organic layer was removed to a vial using a Pasteur pipette.

3.6.3 Derivatization of extracts

Derivatization with diazomethane was used to create methyl esters of carboxylic acids, allowing chromatographic detection of acidic metabolites. Extracts were dried under a nitrogen stream, and then exposed to a solution of diazomethane in diethyl ether (generated using a Diazald kit from Aldrich) for 1 h at room temperature (about 20°C). The ether and residual diazomethane were evaporated under a nitrogen stream, and the sample redissolved in dichloromethane for analysis.

3.6.4 Analysis of culture extracts

Culture extracts were analysed for organic sulfur compounds by GC using a Hewlett Packard 5890 series GC equipped with a 30-m DB-5 capillary column (J&W Scientific, Folsom, CA) and both a flame ionization detector and a flame photometric detector (GC-FID/FPD), or by GC using a Hewlett Packard 5890 series GC equipped with a 25-m HP-1 capillary column (Agilent Technologies, Wilmington, DE) and a FID (GC-FID). Sample standard curves and calculations are shown in Appendix A.

Metabolites were initially identified in culture extracts by low-resolution electron impact (EI) MS using a Hewlett-Packard 5890 series II GC with a 5970 series mass selective detector following a 30-m DB-5 capillary column (J&W Scientific). Further GC-MS (EI), GC-MS with ammonium chemical ionization (NH_4^+ CI), and high-resolution probe MS were performed in the Mass Spectrometry Laboratory, and GC with Fourier-transform infrared detection (GC-FTIR) was performed in the Spectral Services Laboratory, both in the Department of Chemistry, University of Alberta.

3.6.5 Quantitation of acetate

One-millilitre subsamples of culture medium were taken at various times during incubation and frozen at -20°C. To perform acetate analysis, frozen subsamples of culture medium were thawed to room temperature, and diluted if necessary to an acetate concentration between 100 and 200 mg L^{-1}. Ten microlitres of 4 M H_3PO_4 was added to 90 μL of diluted sample for analysis by GC. Acetate was quantified in the prepared samples by GC-FID using a Hewlett-Packard 5890 series GC with a 30-m DB-FFAP capillary column (Agilent Technologies). A sample standard curve is shown in Appendix A.

3.7 Degradation of Lloydminster heavy crude oil in shake flasks

3.7.1 Preparation of flasks

Lloydminster heavy crude oil (#83-02) was weighed (25.13 g) and dissolved in dichloromethane. The solution was filtered through pre-weighed 0.22 μm filters to remove mineral solids, and the filters were dried in a vacuum oven at around 80°C for 3 days and re-weighed. The filtrate was made up with dichloromethane to a total volume of 500 mL in a volumetric flask and 20 mL was added to acid-washed, baffled, 500-mL Erlenmeyer flasks. The remaining filtered oil solution was poured into a glass sample jar and left open in a fume hood to allow the dichloromethane to evaporate, then stored for later analyses. The flasks were left open in a fume hood for three days to allow the solvent to evaporate. SFMM+1% glycerol was added, and then the flasks were closed with foam plugs and aluminum foil and autoclaved. After cooling, the vitamin solution was added, as well as benzyl sulfide in methanol for the spiked controls. The specific compositions of the different flasks are given in Table 3.6.

Table 3.6. Sample compositions for testing the degradation of Lloydminster heavy crude oil in shake flasks

Sample (number of flasks)	Medium	Benzyl sulfide (in methanol)	Phosphate buffer	Live cell suspension	Killed cell suspension
JVH1 live test cultures (3)	180 mL	–	–	20 mL	–
Killed cell controls (3)	100 mL	–	–	–	100 mL
Cell-free sterile controls (3)	180 mL	–	20 mL	–	–
Spiked controls, JVH1 live test cultures (1)	180 mL	2.5 mg	–	20 mL	–
Spiked controls, cell-free sterile controls (1)	180 mL	2.5 mg	20 mL	–	–

3.7.2 Inoculum and incubation

Rhodococcus sp. strain JVH1 was streaked from frozen stocks onto PCA and incubated at 28°C in the dark. After 1 week, 5 mL of phosphate buffer was added to the plate and the biomass was suspended using a sterile glass rod. One millilitre of the cell suspension was added to 200 mL of TSB in four 500-mL Erlenmeyer flasks, which were incubated on a rotary shaker at 140 rpm in the dark for 2 days. Two of the grown cultures were harvested by centrifugation, washed three times and resuspended in an equal volume of phosphate buffer. The final suspensions were pooled and dispensed to each live culture flask (Table 3.6). The remaining cell suspension was killed by autoclaving before dispensing to each killed-cell control bottle (Table 3.6). All the flasks were incubated on a rotary shaker at 140 rpm in the dark at 28°C.

3.7.3 Extraction

After incubation, the pH was measured using pH strips. The contents of each bottle were poured into PTFE centrifuge bottles. Dichloromethane was used to rinse the culture bottles into the centrifuge bottles (approximately 20 mL). The mixtures were then separated by centrifugation for 30 min at 4000×*g*. The aqueous phase was transferred back into the culture flask, and the organic phase was transferred using glass pipettes into clean, preweighed glass sample jars. The centrifuge bottles were rinsed with dichloromethane into the sample jars, and the jars were left open in a fume hood to allow all the solvent to evaporate before weighing the final extracts by difference.

3.7.4 Preparation for analysis

Each individual extract, the 83-02 crude oil, and the filtered oil were tested for sulfur content, and then the triplicate extracts were diluted in dichloromethane, pooled, and the solvent was again allowed to evaporate before further analysis. One of the killed cell controls was contaminated and was not pooled with the other two killed control extracts.

Approximately 0.2 g of each pooled extract and of the crude and filtered oils was partially fractionated by column chromatography using a modification of the method of Fedorak and Westlake [42] to recover the saturates and aromatics fractions for analysis by GC-FID/FPD. Further fractionation to elute the resins and asphaltenes was not performed. To prepare the columns, 8 g of silica was activated overnight in an oven at 125°C. After cooling for around 10 min, dichloromethane was added to the silica in a beaker and the slurry was swirled until no more air bubbles were visible. The slurry was poured into a glass chromatography column plugged with glass wool and pre-filled with dichloromethane. The beaker was rinsed with dichloromethane into the column. The silica was allowed to settle for 45 min, and then sand was poured onto the top of the silica to a depth of around 0.5 cm. The dichloromethane was drained to just above the level of the sand, 60 mL of *n*-pentane was added, and the column was again drained to just above the level of the sand. *n*-Pentane was added to the oil sample (around 2 mL), the mixture was added to the top of the column, and the column was drained to the level of the sand. The column was developed with 10 mL of *n*-pentane, 20 mL of 20 % dichloromethane in *n*-pentane, and 25 mL of 50 % dichloromethane in *n*-pentane. After the first two additions, the column was drained to the level of the sand and the fraction was collected. After the third addition, the column was allowed to drain completely and the fraction was collected. The first fraction (the void volume) was discarded, the second fraction contained saturates, and the third fraction contained aromatics (and benzyl sulfide in the spiked samples). The saturate and aromatic fractions were concentrated by rotary evaporation at reduced pressure and then under a nitrogen stream, and finally dissolved in dichloromethane for GC-FID/FPD analysis.

3.7.5 Analytical methods

Total sulfur content for the oil samples was determined using an Antek 9000NS Nitrogen/Sulfur Analyzer (Antek Instruments, Houston, TX) with dichloromethane as the solvent and dibenzothiophene as the calibration standard. GC-FID/FPD was performed using a Hewlett Packard 5890 series GC equipped with a 30-m DB-5 column (J&W Scientific, Folsom, CA).

Dynamic rheological characterizations (viscosity measurements) were made by Dr. Bin Lin from the Department of Chemical and Materials Engineering, University of Alberta, using a Rheometrics RMS800 rheometer with a 25 mm parallel plate fixture operated at 100 % strain. Each sample was tested twice at 25°C and twice at 50°C over a range of shear rates from 0.10 s^{-1} to 100 s^{-1}. Access to this instrument was provided by Dr. Michael Williams from the Department of Chemical and Materials Engineering, University of Alberta.

4. Enrichment and screening of bacteria active towards aliphatic sulfur compounds under carbon- or sulfur-limited conditions *

4.1 Introduction

Commercially available model compounds were selected to represent the aliphatic sulfur groups found in asphaltenic molecules. These compounds were incubated with soil and water samples to enrich for microorganisms active towards aliphatic sulfides, with the goal of isolating potential biocatalysts for biological oil upgrading. The ideal biocatalyst would effect selective carbon-sulfur bond cleavage in a broad range of aliphatic sulfides, but would not attack aromatic sulfur compounds such as dibenzothiophene. Also, the ideal biocatalyst would not degrade the hydrocarbon portion of the compounds, which would reduce the fuel value of the oil.

Selected bacterial isolates were characterized with respect to their substrate ranges for both sulfur and carbon sources, as well as through identification of the metabolites of aliphatic sulfide degradation. Although none of the isolates studied were ideal for the upgrading application, several mechanisms of degradation were identified including sulfur-specific cleavage and desulfurization of the model compounds. This chapter describes the biotransformations identified for the model aliphatic sulfides, showing a diversity of bacterial activities towards this class of compound.

4.2 Selection of model compounds

Three model compounds were used in enrichment cultures to select for bacteria capable of aliphatic sulfide degradation (Figure 4.1). *n*-Octyl sulfide and *n*-octadecyl sulfide both provide a sulfide bond bounded by long aliphatic hydrocarbon chains, as is expected in the asphaltenes (Figure 2.2B). 1,4-Dithiane is a non-aromatic, sulfur-containing heterocyclic compound. Although a much smaller molecule than the two *n*-alkyl sulfides, the cyclic structure of 1,4-dithiane does not provide any terminal alkyl or aromatic carbon groups, and the high sulfur-to-carbon-ratio ensures that degradation must proceed through cleavage of the aliphatic carbon-sulfur bonds.

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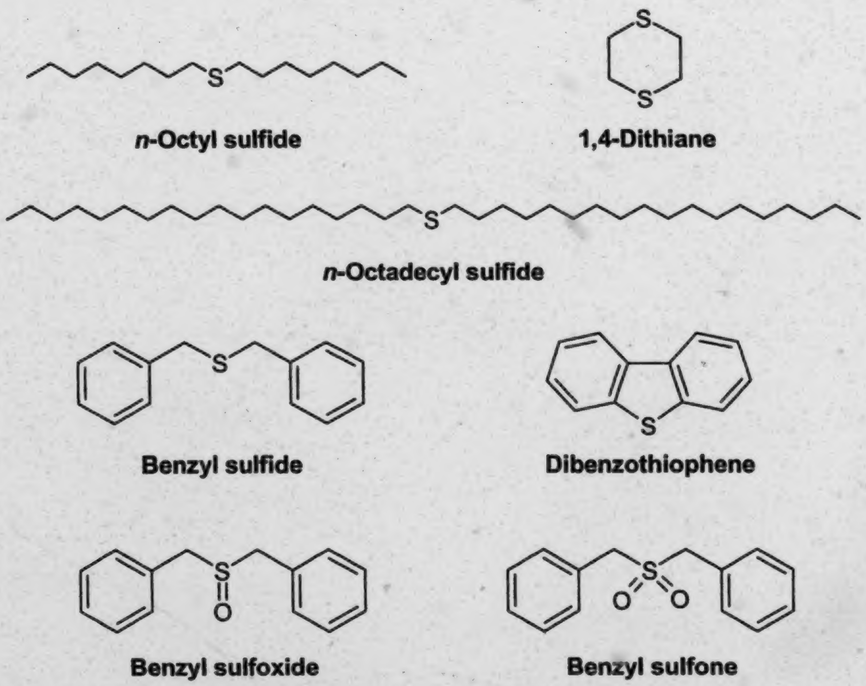


Figure 4.1. Structures of model organic sulfur compounds used as substrates for enrichment and screening.

Benzyl sulfide, benzyl sulfoxide, and benzyl sulfone (Figure 4.1) were used as representative sulfidic (aliphatic) sulfur compounds in the characterization of isolates from sulfur-limited enrichment cultures. Degradation of benzyl sulfide was desirable, as it indicated a potentially broad substrate range for aliphatic sulfides. Benzyl sulfoxide and benzyl sulfone were selected as potential intermediates in the biological desulfurization of benzyl sulfide.

Dibenzothiophene was used as a representative thiophenic (aromatic) sulfur compound (Figure 4.1). Degradation of thiophenic compounds was undesirable, because removal of sulfur from thiophenic structures would not decrease the molecular size of asphaltenic molecules in the intended bio-processing application.

4.3 Enrichment cultures and classification of isolates

4.3.1 Carbon-limited enrichment cultures

Enrichment cultures were successfully established using the linear sulfides *n*-octyl sulfide or *n*-octadecyl sulfide as carbon sources for growth (Table 4.1). Thirty-eight isolates were purified from these carbon-limited enrichment cultures. Of these isolates, nine grew on and degraded both *n*-octyl sulfide and *n*-hexadecane as sole carbon sources. No distinguishing metabolic characteristics were found, so one isolate, designated K7b1, was arbitrarily selected for further study. Enrichment cultures were attempted but could not be maintained with 1,4-dithiane as the carbon source.

Table 4.1. Summary of enrichment and isolation results for carbon-limited enrichment cultures

Sulfur compound used for enrichment	Number of active enrichment cultures *	Total number of isolates purified	Number of active isolates †
n-Octyl sulfide	17	20	9
n-Octadecyl sulfide	17	18	0
1,4-Dithiane	0	-	-

* Assessed as observable increase in turbidity maintained through successive transfers with that substrate
† Assessed as observable growth (turbidity) and loss of substrate

4.3.2 Sulfur-limited enrichment cultures

Enrichment cultures were successfully established using *n*-octyl sulfide, *n*-octadecyl sulfide, or 1,4-dithiane as sulfur sources for growth. The turbidity was quite low in some of these cultures, so the CGA (Section 3.3.4) was used to identify interesting sulfur-limited cultures by comparing growth with a model compound to growth with no added sulfur (negative control) and to growth with sulfate (positive control), using qualitative OD_{600} measurements (Section 3.6.1). In this assay, an active culture would show enhanced growth with the organic sulfur compound compared to the negative control, whereas a non-active culture would show growth equivalent to the negative control (Figure 4.2). The positive control, with sulfate as the sulfur source, was included as a measure of the maximum growth attainable for a specific culture in the CGA medium.

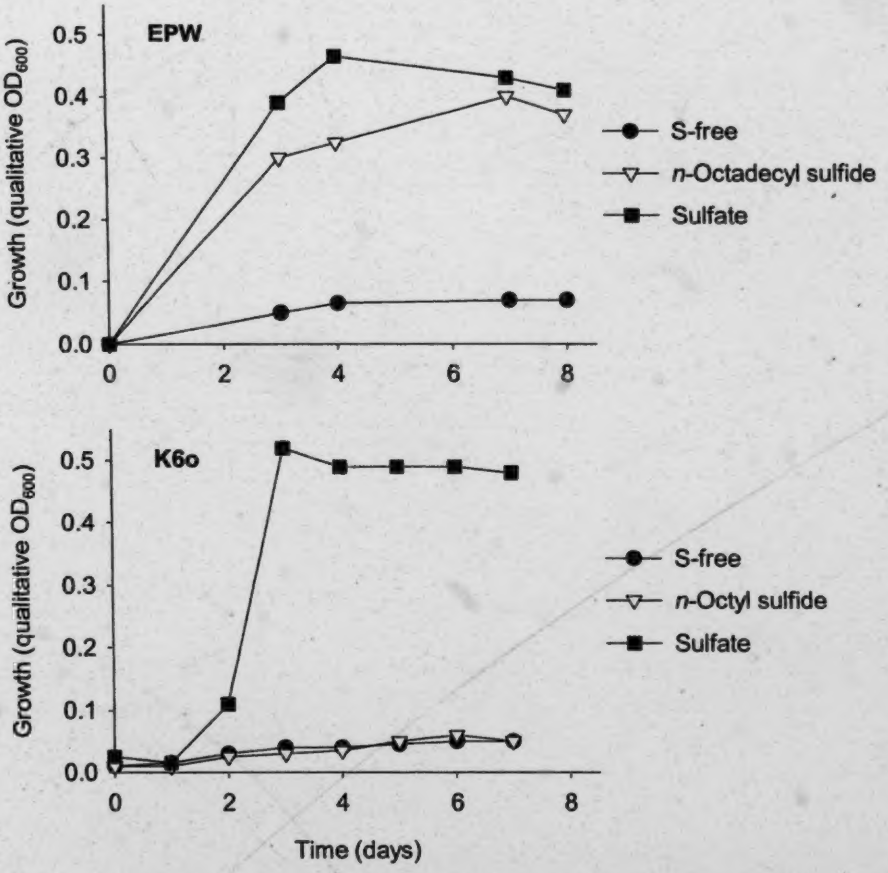


Figure 4.2. Representative CGA data showing a positive result for the EPW enrichment culture screened against *n*-octadecyl sulfide and a negative result for the K6o enrichment culture screened against *n*-octyl sulfide.

The CGA identified 45 mixed enrichment cultures actively growing with *n*-octyl sulfide, *n*-octadecyl sulfide, or 1,4-dithiane as the sole sulfur source (Table 4.2). Two hundred and fifty-five isolates were purified from these enrichment cultures through dilution plating and repeated subculturing on ½PCA. All of the isolates were screened using the CGA to identify individual strains possessing the ability to use the organic sulfur compounds. Three broad classes of isolates were found (Table 4.2). Twenty-two isolates were able to grow using either sulfate or a model compound as the sole sulfur source for growth. A second group was able to grow with sulfate, but not with an organic sulfur source. The third group did not grow in the CGA medium either with sulfate or with an organic sulfur source.

Table 4.2. Summary of CGA results for sulfur-limited enrichment cultures and isolates purified from sulfur-limited enrichment cultures

Sulfur compound used for enrichment	Number of active enrichment cultures	Total number of isolates purified	Isolates growing with sulfate or organic sulfur	Isolates only growing with sulfate	Isolates unable to grow in CGA medium
n-Octyl sulfide	14	77	10	38	29
n-Octadecyl sulfide	19	110	3	61	46
1,4-Dithiane	12	68	9	40	19
Total	45	255	22	139	94

4.3.3 Screening and selection of active isolates from sulfur-limited enrichment cultures

A common feature of bacteria able to use organic sulfur sources is repression of desulfurization activity in the presence of sulfate [142, 159]. The response of the 22 active isolates to sulfate was therefore chosen as a rapid screen for sulfur-specific activity. Growth of the culture and loss of either *n*-octyl sulfide (used for isolates from both *n*-octyl sulfide and *n*-octadecyl sulfide enrichment cultures) or 1,4-dithiane were measured in the presence of acetate alone, acetate and sulfate, and sulfate alone. Isolates with sulfur-specific activity were expected to show growth and to degrade the organic sulfur compound with only acetate present (use of the organic sulfur compound as a sulfur source), growth but no loss of substrate with both acetate and sulfate present (use of sulfate as a sulfur source and repression of desulfurization of the organic sulfur compound by sulfate), and no growth or loss with only sulfate present (no degradation of the organic sulfur compound as a carbon source).

The nine isolates from 1,4-dithiane enrichment cultures all showed the desired pattern of growth and substrate loss (Figure 4.3). Three different patterns of growth and loss were observed among the 13 isolates tested with *n*-octyl sulfide (representative loss results are shown in Figure 4.3):

1. Four Gram-positive isolates showed loss of *n*-octyl sulfide under all conditions, but growth and loss were reduced when only sulfate and *n*-octyl sulfide were present. This pattern indicated poor ability to use *n*-octyl sulfide as a carbon source.
2. Four Gram-positive isolates showed similar substrate loss under all three conditions, despite variations in growth. This pattern suggested non-specific, non-growth related biotransformation of *n*-octyl sulfide.
3. Five Gram-negative isolates did not grow, but completely removed *n*-octyl sulfide in the presence of sulfate alone. They showed growth and similar but incomplete loss in the other two conditions. This pattern again appeared to be non-specific and non-growth related.

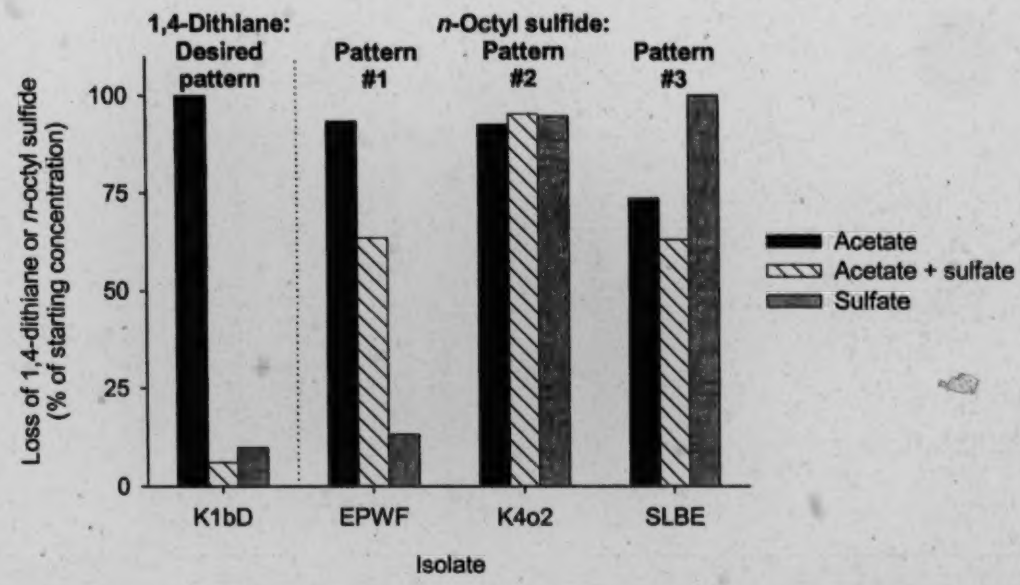


Figure 4.3. **Representative results of screening for sulfur-specific activity showing different patterns of 1,4-dithiane and *n*-octyl sulfide loss in the presence of acetate alone, acetate and sulfate, or sulfate alone as additional carbon or sulfur sources (these patterns and their relation to culture growth are discussed in the text).**

None of these patterns matched the desirable combination described previously. Pattern 1 was the most interesting because of the lack of degradation of *n*-octyl sulfide as a carbon source.

The 22 isolates were further grouped in three categories (Table 4.3), based on their ability to degrade *n*-alkanes (*n*-hexadecane and *n*-eicosane) as carbon sources and to use dibenzothiophene as a sulfur source. Both of these characteristics were undesirable, because degradation of *n*-alkanes would reduce the fuel value of the oil being processed, and desulfurization of thiophenes would not decrease the molecular size of asphaltenic material. One isolate from each category was selected for further study (Table 4.3). Isolate K1oA was the only isolate that did not degrade *n*-alkanes, and was also the only Gram-negative isolate from the 1,4-dithiane enrichment cultures. The remaining eight isolates from 1,4-dithiane enrichment cultures all used dibenzothiophene as a sulfur source, but also demonstrated the desired sulfur-specific activity towards 1,4-dithiane. No distinguishing metabolic characteristics were found, therefore isolate K1bD was chosen based on a readily distinguishable colonial morphology on PCA. Isolate EPWF was the only isolate from the *n*-alkyl sulfide enrichment cultures to show any evidence for sulfate regulation (reduced degradation of *n*-octyl sulfide in the presence of sulfate, Figure 4.3), which is an indicator of sulfur-specific activity, and so was selected as the representative isolate from the group degrading *n*-alkanes but not using dibenzothiophene as a sulfur source.

Table 4.3. Bacterial isolates from sulfur-limited enrichment cultures growing with at least one model compound as sole sulfur source, grouped according to carbon and sulfur source

Test characteristics		Number of isolates	Representative isolate
n-Alkane degradation as carbon source *	Dibenzothiophene utilization as sulfur source		
-	-	1	*Pseudomonas* sp. strain K1oA
+	-	12	*R. erythropolis* strain EPWF
+	+	9	*Rhodococcus* sp. strain K1bD

* *n*-Hexadecane and *n*-eicosane

4.3.4 Identification and characterization of selected isolates

The Gram reaction, assayed using 3 % KOH, showed that isolate K1oA was Gram-negative, and isolates K7b1, K1bD, and EPWF were Gram-positive. The four isolates were then identified using Biolog GN2 and GP2 MicroPlates and the MicroLog database. K1oA was identified at the genus level as a species of *Pseudomonas* (similarity of 0.35 to *P. fluorescens* biotype A after 24 h incubation). K7b1, K1bD, and EPWF were identified as strains of *R. erythropolis* (probabilities of 99 %, 100 %, and 87 %, respectively, after 48 h incubation).

EPWF was the most promising isolate because it oxidized a variety of aliphatic sulfides but not dibenzothiophene as sulfur sources, and was therefore selected for further testing. EPWF was a Gram-positive, catalase positive, acid-fast bacterium with rod-shaped cells. No branching growth was observed. EPWF was confirmed as a strain of *R. erythropolis* through sequencing of the full 16S rRNA gene (total sequence length: 1513 base pairs). Based on the 16S rRNA gene sequence, EPWF showed greater than 99 % similarity to *R. erythropolis* strain NVI 00/50/6670 (GenBank accession number AY147846, 1477 matches out of 1479 aligned base pairs) and to *N. simplex* ATCC 13260 (reclassified as *R. erythropolis* ATCC 13260 [174], GenBank accession number U81990, 1472 matches out of 1473 aligned base pairs).

Since the ability of isolate EPWF to degrade *n*-alkanes was undesirable, EPWF was further tested for the ability to degrade a variety of compounds representing different structures found in oil using spray, spread, or vapour plates. EPWF was able to grow on linear alkanes (*n*-octane, *n*-hexadecane, *n*-eicosane), the isoprenoid alkane pristane, and monoterminally substituted alkanes (*n*-dodecylcyclohexane, 1-phenyldodecane). Growth was not observed on cyclic alkanes (cycloheptane, cyclooctane), the highly branched alkane HMN, or polycyclic aromatic compounds (naphthalene, anthracene, phenanthrene, dibenzothiophene, carbazole).

The hydrophobicity of the three isolates from sulfur-limited enrichment cultures was tested using the BATH assay [135]. As shown in Table 4.4, all three isolates showed some adhesion to *n*-hexadecane, although none were as hydrophobic as the positive control organism, *A. venetianus* strain RAG-1.

Table 4.4. BATH assay results for three isolates from sulfur-limited enrichment cultures

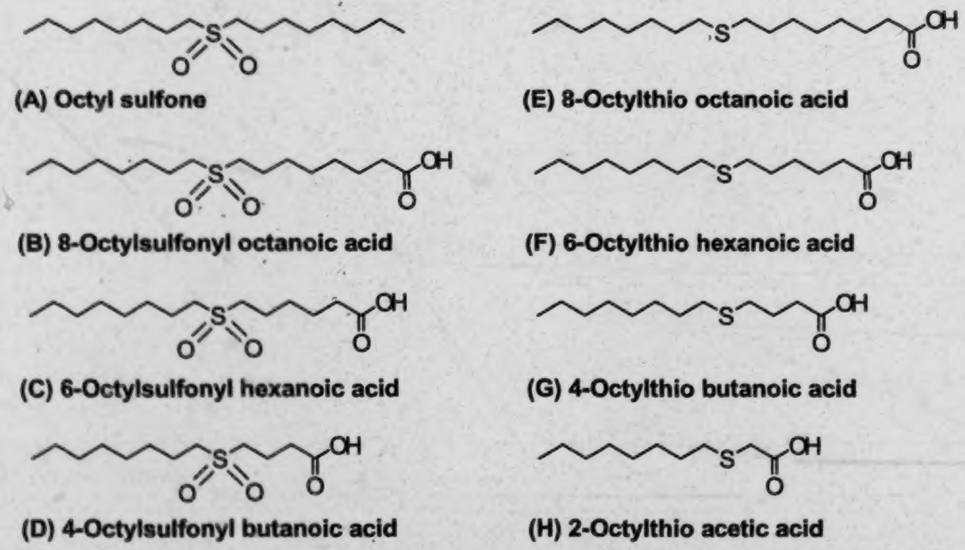
Organism	Initial OD_{600}	Final OD_{600} *	Percent adhesion to hydrocarbon
R. erythropolis strain EPWF	0.324	0.032	90
Pseudomonas sp. strain K1oA	0.446	0.146	67
Rhodococcus sp. strain K1bD	0.450	0.213	53
A. venetianus strain RAG-1 (positive control)	0.327	0.000	100
P. fluorescens strain LP6a (negative control)	0.375	0.377	0

* after mixing 1.2 mL of cell suspension with 2 mL of *n*-hexadecane

4.4 Use of *n*-octyl sulfide as a sulfur or carbon source

n-Octyl sulfide was subject to terminal oxidation and degradation by *n*-alkane-degrading isolates from both carbon-limited and sulfur-limited enrichment cultures, as well as sulfur oxidation by all four isolates. All products identified are shown in Figure 4.4. (Mass and FTIR spectra used for identification of metabolites are shown in Appendix B.)

Rhodococcus sp. strain K7b1 and the other *n*-hexadecane-degrading isolates from carbon-limited enrichment cultures accumulated two metabolites after growth with *n*-octyl sulfide as the sole carbon source. One metabolite is proposed to be 2-octylthio acetic acid (Compound H, Figure 4.4). GC-MS (EI) showed a molecular weight of 204 with a major fragment at *m/z* 145. The molecular ion increased to *m/z* 218 after derivatization with diazomethane, showing the presence of a carboxylic acid. High resolution probe MS indicated the molecular formula to be $C_{10}H_{20}O_2S$ for the parent compound, and $C_8H_{17}S$ for the fragment at *m/z* 145 (consistent with cleavage of one carbon-sulfur bond). GC-FTIR showed the presence of a carboxylic acid with bands at 1782 cm^{-1} (C=O) and 3576 cm^{-1} (O-H) [118].



(A) Octyl sulfone

(B) 8-Octylsulfonyl octanoic acid

(C) 6-Octylsulfonyl hexanoic acid

(D) 4-Octylsulfonyl butanoic acid

(E) 8-Octylthio octanoic acid

(F) 6-Octylthio hexanoic acid

(G) 4-Octylthio butanoic acid

(H) 2-Octylthio acetic acid

Figure 4.4. Biotransformation and biodegradation products identified in cultures of *Rhodococcus* sp. strain K7b1 (A and H), *Rhodococcus* sp. strain K1bD (A and D), *R. erythropolis* strain EPWF (A-H), and *Pseudomonas* sp. strain K1oA (A) incubated with *n*-octyl sulfide as the sole carbon (K7b1) or sulfur source.

The other metabolite is proposed to be octyl sulfone (Compound A, Figure 4.4). This product did not have a clear molecular ion after GC-MS (EI), but did have a major fragment with an *m/z* of 179. GC-MS (NH_4^+ CI) showed the molecular weight to be 290 (*m/z* 308 for $[M+18]^+$). High resolution probe MS gave a molecular formula of $C_{16}H_{34}O_2S$ for the compound and $C_8H_{19}O_2S$ for the fragment at *m/z* 179. These results are consistent with long-chain alkyl sulfones, which have weak molecular ions and which undergo cleavage at the sulfur atom and double hydrogen rearrangement under electron impact [16]. GC-FTIR confirmed the presence of a sulfone with bands at 1337 cm^{-1} and 1139 cm^{-1} [145].

A different pattern of transformation was found for the *n*-alkane-degrading isolates from sulfur-limited enrichment cultures. The primary metabolites were proposed to be 4-octylsulfonyl butanoic acid and octyl sulfone (Compounds D and A, respectively, Figure 4.4), found in cultures of *Rhodococcus* sp. strain K1bD and *R. erythropolis* strain EPWF. Traces of 6-octylsulfonyl hexanoic acid and 8-octylsulfonyl octanoic acid

(Compounds C and B, respectively, Figure 4.4) were also found in cultures of *R. erythropolis* strain EPWF. Octylsulfonyl alkanoic acids were identified through analysis of their methyl esters by GC-MS (EI and NH_4^+ CI). These compounds were characterized by a weak (or absent) molecular ion under EI, and a major fragment with a mass consistent with the formula $(CH_2)_nCO_2CH_3$ (n=3, 5, or 7), formed by cleavage of the carbon-sulfur bond on the same side as the carboxylic acid. The two longest acids also had a major fragment at 179, analogous to octyl sulfone. No shorter acids, acids with an odd number of carbon atoms, diterminal oxidation products, or acids with the sulfur atom still reduced were found.

R. erythropolis strain EPWF was grown with an ion-exchange resin in the culture medium to trap acidic metabolites, allowing the pathway of *n*-octyl sulfide degradation to be assessed. Products detected by GC-MS are proposed to be octyl sulfone, 8-octylsulfonyl octanoic acid, 8-octylthio octanoic acid, 6-octylthio hexanoic acid, 4-octylthio butanoic acid, and 2-octylthio acetic acid (Compounds A, B, and E-H, respectively, Figure 4.4). The fragmentation patterns of the methyl esters of the proposed alkylthio alkanoic acids are given in Table 4.5 according to the characteristic fragmentation of this class of compound reported by Christie *et al.* [26] (Figure 4.5). Although a control experiment showed that this method would have recovered octanoic acid, no shorter acids either with or without the sulfur atom were detected in the culture extract.

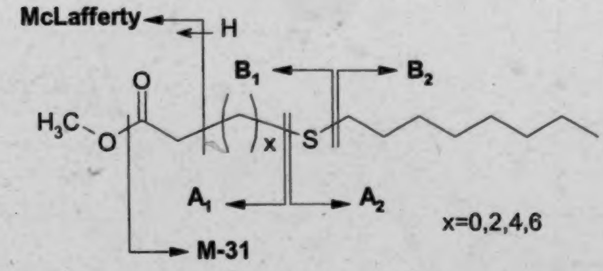


Figure 4.5. Characteristic electron impact fragmentation pattern of alkylthio alkanoic acid methyl esters (adapted from Christie *et al.* [26]), shown for octylthio alkanoic acid methyl esters.

Table 4.5. **Mass spectral data for the methyl esters of octylthio alkanoic acids formed from *n*-octyl sulfide by *R. erythropolis* strain EPWF, based on the characteristic ions identified by Christie *et al.* [26]**

Compound	Base peak	Characteristic ions, *m/z* (abundance)							
		M$^+$	M-31	McLafferty ion	A$_1$	A$_2$	B$_1$	B$_2$	A$_1$-32
8-Octylthio octanoic acid	55	302 (4)	271 (2)	74 (29)	157 (31)	145 (26)	189 (2)	113 (7)	125 (17)
6-Octylthio hexanoic acid	55	274 *	243 (2)	74 (31)	129 (39)	145 (24)	161 (2)	113 (2)	97 (29)
4-Octylthio butanoic acid	59	246 (11)	215 (4)	74 (61)	101 (74)	145 (24)	134 † (10)	113 *	69 (63)
2-Octylthio acetic acid	69	218 (7)	187 *	74 (87)	73 (16)	145 (43)	106 † (7)	113 *	41 *

* Ions not observed in the mass spectrum
† At B$_1$+1

Pseudomonas sp. strain K1oA could not grow with *n*-octyl sulfide as a sulfur source, but did produce a small amount of octyl sulfone (compound A, Figure 4.4). No terminal degradation products were found.

4.5 Use of *n*-octadecyl sulfide

Degradation of *n*-octadecyl sulfide as a sulfur source was not detectable in cultures of *R. erythropolis* strain EPWF, even though some growth on *n*-octadecyl sulfide was observed during the CGA screening of this isolate. This observation suggested that the lack of degradation might be due to limited mass transfer from *n*-octadecyl sulfide crystals to the cells. *n*-Octadecyl sulfide was dissolved in HMN as a non-toxic, non-biodegradable carrier solvent, and the solution was added to the cultures at a 1 % volume ratio. This approach allowed complete removal of *n*-octadecyl sulfide as a sulfur source (Figure 4.6), as well as an observable increase in biomass in the culture flasks compared to sulfur-free negative controls. Growth was not measured directly due to clumping of the biomass and adhesion to the organic phase. No metabolites were detected in the culture extracts before or after derivatization with diazomethane.

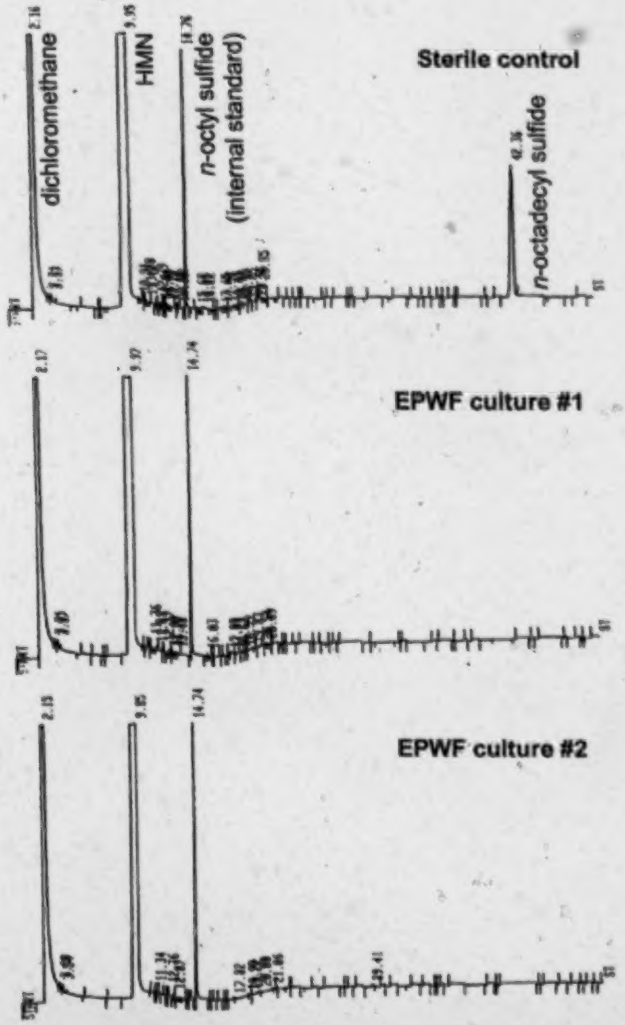


Figure 4.6. GC-FID chromatograms of dichloromethane extracts showing complete loss of *n*-octadecyl sulfide when provided in HMN as a sulfur source for replicate cultures of *R. erythropolis* strain EPWF.

4.6 Use of 1,4-dithiane as a sulfur source

Both the commercial preparation and recrystallized 1,4-dithiane were used as a sulfur source by all three isolates from sulfur-limited enrichment cultures. The effect of sulfate concentration on the degradation of 1,4-dithiane was tested as an indicator of sulfur-specific activity. The extent of substrate loss was diminished in the presence of sulfate (Figure 4.7), although this effect differed among the isolates. The addition of 5 mmol sulfate L^{-1} almost completely prevented the degradation of 1,4-dithiane by *Rhodococcus* sp. strain K1bD, but had only a small effect on degradation by *R. erythropolis* strain EPWF. No metabolites were found in 1,4-dithiane-degrading culture extracts.

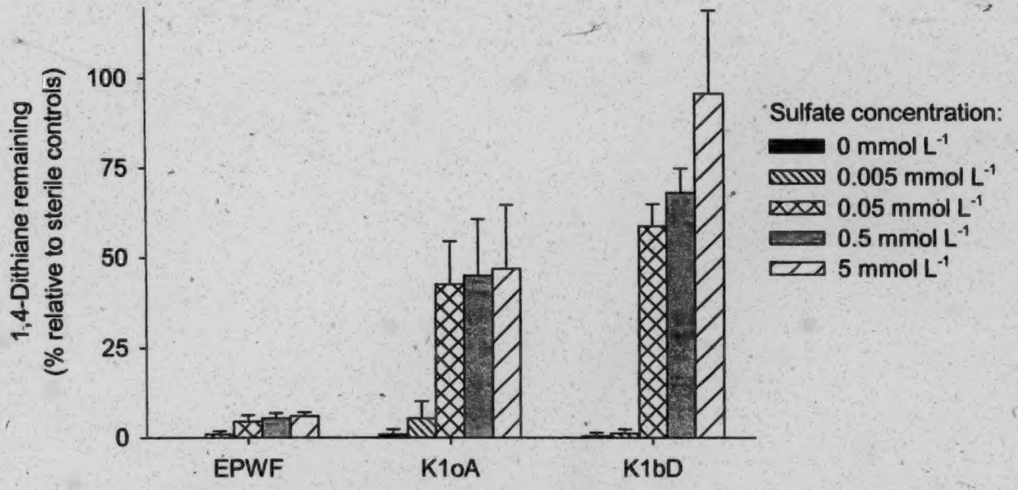


Figure 4.7. Effect of sulfate concentration on the biodegradation of 1,4-dithiane (0.5 mmol L^{-1}) by *R. erythropolis* strain EPWF, *Pseudomonas* sp. strain K1oA, and *Rhodococcus* sp. strain K1bD (no 1,4-dithiane was detected in cultures of EPWF with no added sulfate; average + standard deviation, n=3).

4.7 Benzyl sulfide oxidation and desulfurization

In initial experiments, *R. erythropolis* strain EPWF grew with benzyl sulfide and benzyl sulfoxide as sole sulfur sources. Loss of benzyl sulfide and benzyl sulfoxide, however, was accompanied by nearly stoichiometric accumulation of benzyl sulfone (identified through comparison of the GC retention time and of the mass spectrum to an authentic standard), so these compounds could not have been used as sulfur sources for growth. In addition, benzyl sulfone did not support growth and was not degraded as a sulfur source. To determine whether sulfur-containing contaminants were supporting the observed growth of EPWF, the commercial preparation of benzyl sulfide was further purified by recrystallization. The purified benzyl sulfide did not support growth, indicating that an unidentified contaminant in the original chemical stock was serving as the sulfur source for this organism. Presumably contaminants in the benzyl sulfoxide preparation were also responsible for the observed growth on this substrate, but this hypothesis was not tested. The degree of conversion of purified benzyl sulfide to benzyl sulfone was not affected by sulfate (Figure 4.8).

In contrast, *Rhodococcus* sp. strain K1bD used purified benzyl sulfide as a sulfur source for growth with non-stoichiometric accumulation of benzyl sulfoxide and sulfone in the culture medium. Two other trace metabolites found in culture extracts had the same GC-FID retention times as benzyl alcohol and benzoic acid both before and after derivatization with diazomethane. The presence of sulfate resulted in greater accumulation of benzyl sulfone (Figure 4.8).

Pseudomonas sp. strain K1oA could not grow with benzyl sulfide as sole sulfur source, and no benzyl sulfone was detected in the absence or presence of sulfate (Figure 4.8).

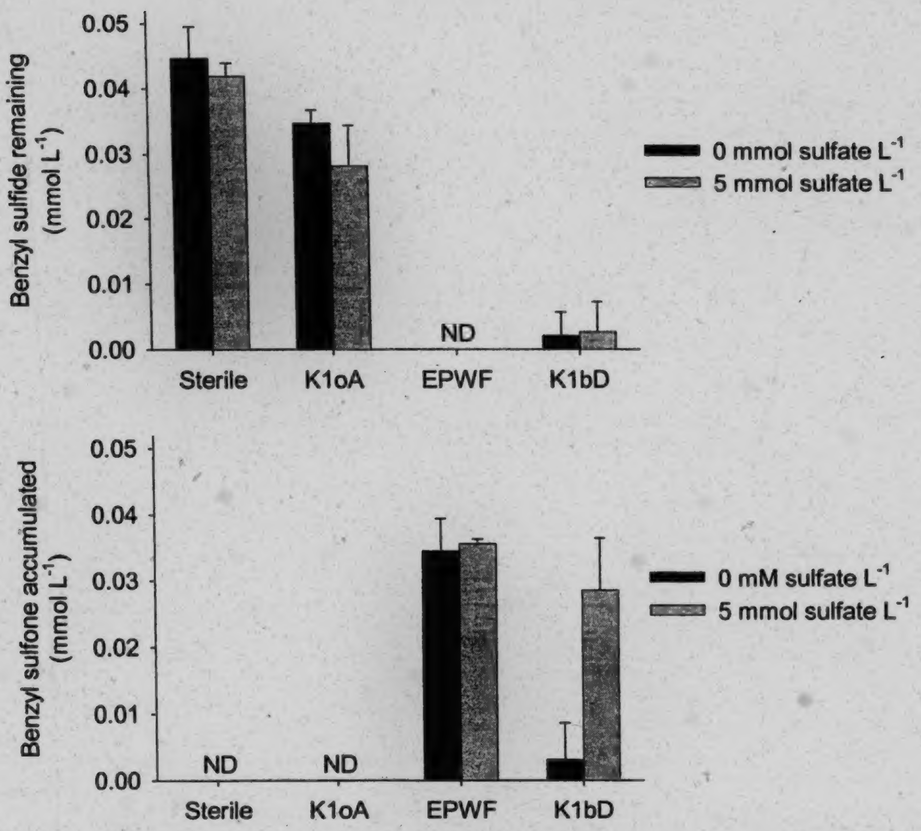


Figure 4.8. **Effect of sulfate on the biodegradation of benzyl sulfide and the accumulation of benzyl sulfone in cultures of *Pseudomonas* sp. strain K1oA, *R. erythropolis* strain EPWF, and *Rhodococcus* sp. strain K1bD incubated with benzyl sulfide as the sole organic sulfur source (ND – none detected; average + standard deviation, n=3).**

4.8 Use of dibenzothiophene as a sulfur source

Sulfur-limited enrichment cultures with aliphatic sulfides also led to strains active towards dibenzothiophene. Eight strains from 1,4-dithiane enrichment cultures and one from an *n*-octadecyl sulfide enrichment culture used dibenzothiophene as a sulfur source (Table 4.3). The only metabolite found was 2-hydroxybiphenyl, the end product of the 4S pathway for dibenzothiophene biodesulfurization [49], identified by comparison of the mass spectrum to published spectra. A preference for aliphatic sulfides over thiophenic compounds was desired, because only cleavage of the aliphatic sulfur bridges would reduce the molecular size of asphaltenes. The selectivity of the desulfurization system was tested by growing *Rhodococcus* sp. strain K1bD with dibenzothiophene and 1,4-dithiane both separately and in combination. Although each compound was readily degraded as the sole sulfur source, only dibenzothiophene was degraded when both compounds were provided in the growth medium (Figure 4.9).

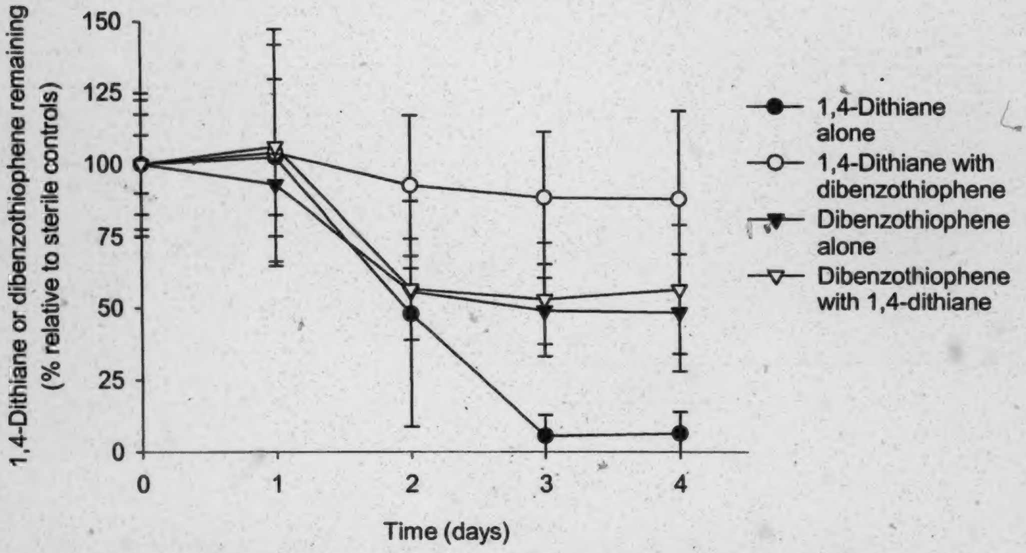


Figure 4.9. Degradation by *Rhodococcus* sp. strain K1bD of 1,4-dithiane or dibenzothiophene when provided as sulfur sources separately or together (each compound provided at 0.25 mmol S L^{-1}).

4.9 Discussion and conclusions

Enrichment cultures were successfully established using the linear sulfides *n*-octyl sulfide or *n*-octadecyl sulfide as carbon sources or as sulfur sources for growth. Active enrichment cultures were also obtained using the cyclic sulfide 1,4-dithiane as a sulfur source. Enrichment cultures were started from several inocula with 1,4-dithiane as the carbon source, but these cultures all failed to grow and were abandoned. The microbial populations in the environmental samples tested may not have had the metabolic ability to utilize the carbon from this compound. Alternately, 1,4-dithiane may have been toxic to the cultures at the higher concentrations required to support growth as a carbon source instead of as a sulfur source. Toxicity was not tested separately.

Thirty-one bacterial strains isolated from enrichment cultures were able to transform model compounds representing the aliphatic sulfide bridges found in asphaltenes. Three general types of attack were observed: alkyl chain degradation, allowing use as a carbon source; non-specific sulfur oxidation without carbon-sulfur bond cleavage; and sulfur oxidation with carbon-sulfur bond cleavage, allowing use as a sulfur source. One or more of these pathways was demonstrated by different microorganisms and towards different substrates.

All but one of the active isolates were able to degrade *n*-hexadecane as a carbon source. Three *Rhodococcus* isolates from this group (strains K7b1, K1bD, and EPWF) grown with *n*-octyl sulfide caused sulfur oxidation (producing octyl sulfone) as well as terminal oxidation and degradation (producing octylthio- and octylsulfonyl-alkanoic acids). The identification of octylthio alkanoic acids (Table 4.5) was based on the fragmentation pattern of the methyl ester in the mass spectrum, which followed Christie *et al.* [26] for alkylthio alkanoic acids, as shown in Figure 4.5. Ions were formed through cleavage on both sides of the sulfur atom (A_1, A_2, B_1, and B_2), as well as loss of the methoxy group from the parent compound (M-31) and from the A_1 fragment (A_1-32). The McLafferty rearrangement ion ($m/z = 74$) was formed through γ-hydrogen rearrangement and β-cleavage [107]. The low abundance or absence of the M-31 and B_2 ions and the observation of B_1+1 ions for 2-octylthio acetic acid and 4-octylthio butanoic acid are consistent with the methyl esters of 3- and 5-thiastearate (methyl esters of 2-pentadecylthio acetic acid, and 4-tridecylthio butanoic acid, respectively) [26].

Jenisch-Anton *et al.* [66, 67] identified alcohols and odd-numbered side chains from the degradation of both phytanyl octadecyl ether and phytanyl octadecyl sulfide (Figure 2.5), indicating that subterminal cleavage reactions other than β-oxidation occurred in their cultures. In contrast, all of the metabolites identified in this work from alkyl chain degradation of *n*-octyl sulfide were monocarboxylic acids retaining an even number of carbon atoms in the degraded side chain. This result indicated mono-terminal- and β-oxidation reactions analogous to the aerobic biodegradation of *n*-alkanes [6], and suggests that terminal degradation of *n*-octyl sulfide is catalysed by the alkane hydroxylase complex of these bacteria.

Control experiments with hexanedioic acid and thiophene-2,5-dicarboxylic acid showed that the extraction method used would not recover dicarboxylic acids if they were formed from *n*-octyl sulfide. The accumulation of the sulfur-containing monocarboxylic acids, however, suggests that further degradation reactions did not occur in these cultures.

Complete mineralization of *n*-octyl sulfide was apparently blocked by the sulfur atom, leading to two different end products depending on the oxidation state of the sulfur atom: 2-octylthio acetic acid, the main metabolite produced by *Rhodococcus* sp. strain K7b1; and 4-octylsulfonyl butanoic acid, the main metabolite produced by *Rhodococcus* sp. strain K1bD and *R. erythropolis* strain EPWF. The presence of a sulfur atom is known to block β-oxidation, and alkylthio acetic acids (3-thia fatty acids) have been used as non-β-oxidizable fatty acid analogues in other studies [10]. The bacteria would therefore require a different enzyme system to continue mineralization past the sulfur atom. Bacteria capable of degrading *n*-alkanes initially degrade the octadecyl side chain of phytanyl octadecyl ether, and can then continue past the ether bond to at least partially degrade the phytanyl portion [67]. In contrast, only the octadecyl side chain of phytanyl octadecyl sulfide is degraded, leaving sulfur-containing metabolites [66]. The shortest metabolites of phytanyl octadecyl sulfide were 2-phytanylthio ethanol and 2-phytanylthio acetic acid (where the sulfur atom is still reduced), and 2-phytanylsulfonyl ethanol and 3-phytanylsulfonyl propanoic acid (where the sulfur atom is oxidized to the sulfone), consistent with the observation that β-oxidation did not occur less than three carbon atoms away from the sulfone group. This specificity would explain the accumulation of 2-octylthio acetic acid and 4-octylsulfonyl butanoic acid in the current study.

Degradation of *n*-octadecyl sulfide should proceed by the same mechanisms as *n*-octyl sulfide due to the structural similarity of these compounds. The products expected to accumulate in cultures of *R. erythropolis* strain EPWF in the absence of an ion exchange resin were 4-octadecylsulfonyl butanoic acid and octadecyl sulfone. Despite complete loss of the starting compound, however, no metabolites were detected in derivatized extracts of cultures grown with *n*-octadecyl sulfide in HMN as the sole sulfur source. Assuming analogous behaviour to 4-octylsulfonyl butanoic acid and octyl sulfone, 4-octadecylsulfonyl butanoic acid and octadecyl sulfone would elute later than *n*-octadecyl sulfide under the GC-FID conditions used. The temperature program may, therefore, not have been long enough or hot enough to elute the metabolites. Another possibility is that the culture was capable of diterminal oxidation of this longer molecule, resulting in dicarboxylic acids that were not extracted from the cultures.

Benzyl alcohol and benzoic acid were identified as metabolites of benzyl sulfide desulfurization by *Rhodococcus* sp. strain K1bD. Benzyl alcohol is presumably the initial cleavage product, and would be readily oxidized to benzoic acid, which would then be used as a carbon source by the culture. This mechanism is analogous to the pathway identified in *Rhodococcus* sp. strain JVH1, which oxidizes and cleaves PFPS to produce 3-pentafluorophenylpropan-1-ol, which is further oxidized to 3-pentafluorophenylpropanoic acid (Figure 2.6) [160]. *Rhodococcus* sp. strain K1bD also uses dibenzothiophene as a sulfur source producing 2-hydroxybiphenyl. In other microorganisms producing 2-hydroxybiphenyl from dibenzothiophene, the hydroxyl group is introduced when the first carbon-sulfur bond is cleaved [49].

Rhodococcus sp. strain K1bD may exhibit two mechanisms for oxidation and degradation of *n*-octyl sulfide. The detection of 4-octylsulfonyl butanoic acid in *Rhodococcus* sp. strain K1bD culture extracts suggests that this compound is the end product of the terminal degradation pathway, as in *R. erythropolis* strain EPWF. The mechanism by which *Rhodococcus* sp. strain K1bD releases the sulfur from *n*-octyl sulfide to support growth was not determined. Because octyl sulfone was produced by the cultures, further reactions may be analogous to the use of benzyl sulfide, producing 1-octanol after carbon-sulfur bond cleavage. 1-Octanol is used as a carbon source by *Rhodococcus* sp. strain K1bD; therefore, this product would not necessarily be detected.

The ability of bacteria to use organic sulfur sources is commonly repressed by inorganic sulfate [142, 159]. Use of a model compound as a sulfur source and regulation of degradation in the presence of sulfate were taken, therefore, as evidence of sulfur-specific activity. In the current study, desulfurization of aliphatic sulfides was regulated by inorganic sulfate and was therefore a sulfur-specific activity. Loss of 1,4-dithiane was regulated by sulfate in all nine isolates from 1,4-dithiane enrichment cultures (including *Rhodococcus* sp. strain K1bD and *Pseudomonas* sp. strain K1oA), as well as in *R. erythropolis* strain EPWF (Figure 4.7). Benzyl sulfone accumulated in *Rhodococcus* sp. strain K1bD cultures grown with benzyl sulfide and sulfate (Figure 4.8), indicating that carbon-sulfur bond cleavage, and subsequent sulfur release, was regulated by inorganic sulfate.

n-Octyl sulfide was oxidized to octyl sulfone by *Rhodococcus* sp. strain K7b1, *Pseudomonas* sp. strain K1oA, and *R. erythropolis* strain EPWF, and benzyl sulfide was oxidized to benzyl sulfone by *R. erythropolis* strain EPWF, although there was no chemical evidence for carbon-sulfur bond cleavage or sulfur release, and these compounds did not support growth as sole sulfur sources. In addition, oxidation of benzyl sulfide to benzyl sulfone was not regulated by sulfate in cultures of *R. erythropolis* strain EPWF and *Rhodococcus* sp. strain K1bD. Therefore, oxidation of aliphatic sulfides to sulfones was interpreted to be a non-specific reaction in these bacteria. Sulfur oxidation is known to be a non-specific reaction catalysed by a variety of enzymes including alkane monooxygenase [69], fungal laccase and cytochrome P450 [162], yeast desaturases [21], and naphthalene dioxygenase [141].

The technique of enrichment using commercially available aliphatic sulfides did not exclusively select for microorganisms exhibiting sulfur-specific attack on aliphatic sulfide bonds. Instead, a range of activities was identified in the bacteria isolated from these enrichment cultures, illustrated schematically in Figure 4.10. The full range of these activities was exemplified by *Rhodococcus* sp. strain K1bD, which showed specific desulfurization of 1,4-dithiane and benzyl sulfone, but also degraded the hydrocarbon portion of *n*-octyl sulfide, exhibited non-specific sulfur oxidation of benzyl sulfide, desulfurized dibenzothiophene through the 4S pathway, and demonstrated selectivity for dibenzothiophene over 1,4-dithiane as a sulfur source. Different subsets of these behaviours were demonstrated by other isolates. The approach of Van Hamme *et al.* [160] is therefore more appropriate for specific enrichment of aliphatic sulfide cleavage activity. Their use of the novel compound PFPS, which is blocked from terminal attack through full fluorination of the phenyl groups, allowed for enrichment and selection of a bacterium exhibiting the desired sulfur-specific cleavage reactions.

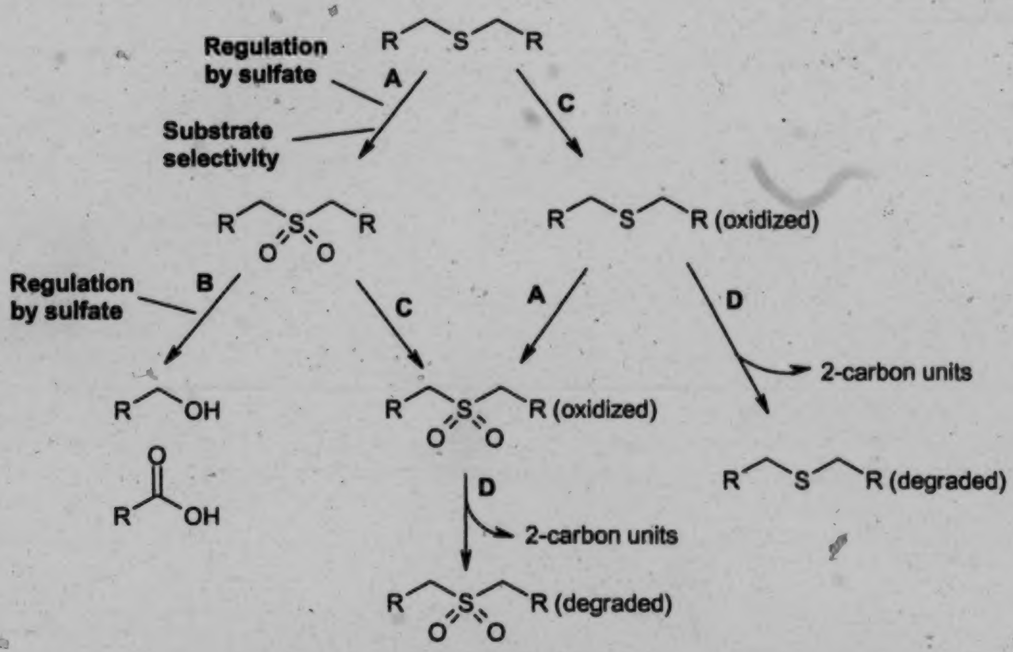

Regulation by sulfate
A
Substrate selectivity
C
R (oxidized)
Regulation by sulfate
B
C
A
D
2-carbon units
R (oxidized)
R (degraded)
D
2-carbon units
R (degraded)

Figure 4.10. Summary of the biotransformations identified in the bacterial biodegradation of aliphatic sulfides, showing (A) sulfur oxidation, (B) desulfurization, (C) oxidation of terminal hydrocarbon groups, and (D) degradation through β-oxidation of terminal hydrocarbon groups.

Enrichment cultures were successful for isolating bacteria active towards model compounds containing aliphatic sulfide bonds. Some of these strains showed sulfur-specific bacterial cleavage of aliphatic carbon-sulfur bonds in a variety of compounds. Identification of this type of reaction is of interest for molecular weight and viscosity reduction in heavy oils, and supports the hypothesis that a biological upgrading process for viscosity reduction in heavy oils may be feasible. Although the desired reactions were identified in some isolates, alternate pathways were also identified, indicating that care must be taken when prospecting for bacteria as potential upgrading catalysts. The regulatory effect of sulfate may also be a significant barrier for sulfur-specific bioprocessing applications. The non-sulfur-specific pathways, which are not affected by inorganic sulfate, may still be useful for biodegradation applications, for example the biotreatment of toxicants such as sulfur mustard, or the bioremediation of sulfur-containing petroleum contaminants.

5. Sulfur- and carbon-source substrate ranges for *Rhodococcus* sp. strain JVH1

5.1 Introduction

Enrichment cultures using commercially-available organic sulfur compounds were not successful for isolating a bacterium with selective, sulfur-specific activity towards aliphatic sulfur compounds (Chapter 4). *Rhodococcus* sp. strain JVH1 [160] was therefore chosen for further studies. JVH1 is capable of using the novel aliphatic sulfur compound PFPS as its sole sulfur source for growth through a sulfur specific pathway analogous to the 4S pathway for dibenzothiophene biodesulfurization (Figure 2.6) [160]. JVH1 was also shown to use a variety of compounds with aliphatic carbon-sulfur bonds as sulfur sources, but not the thiophenic compound dibenzothiophene [160].

The activity of *Rhodococcus* sp. strain JVH1 towards organic sulfur compounds other than PFPS was investigated in more detail, including the testing of additional compounds to fully define the substrate range, identification of metabolites where possible, and analysis of selectivity between different compounds. JVH1 was discovered to use the thiophenic compound benzothiophene as a sulfur source, and studies of the metabolism of benzothiophene and alkylbenzothiophenes are also presented in this chapter. Finally, JVH1 was shown to degrade linear and isoprenoid alkanes as carbon sources for growth, and an attempt was made to control this ability through catabolite repression.

5.2 Substrate range and selectivity among organic sulfur sources

5.2.1 Use of organic sulfur sources

The substrate range of *Rhodococcus* sp. strain JVH1 for sulfidic, thiophenic, and oxidized organic sulfur sources was tested with a wide variety of compounds to replicate and expand the results of Van Hamme *et al.* [160]. Where possible, degradation of the compounds was confirmed through GC-FID analysis, and any metabolites appearing in the chromatograms were identified by GC-MS (Section 3.6.4). These results are summarized in Table 5.1. JVH1 showed a broad substrate range for sulfidic compounds, and not for aromatic sulfur compounds. Phenyl sulfide and benzyl disulfide inhibited even the low level of growth observed in sulfur-free negative controls, measured as turbidity (OD_{600}) directly in the culture tubes. Significant disappearance of these compounds was observed, but no metabolites were detected. The observed loss may have been due to oxidation of the compounds or sorption to the biomass, but was not investigated further because of the inhibitory nature of the compounds.

The three-ring aromatic compounds dibenzothiophene, dibenzothiophene sulfone, and thianthrene did not serve as sulfur sources for growth of JVH1, and growth was equivalent to the sulfur-free negative controls. Growth of cultures with the two-ring aromatic compound benzothiophene, accompanied by over 90 % loss of the compound, was an unexpected result, contradicting the results of Van Hamme *et al.* [160]. Additional tests showed that JVH1 grew readily with benzothiophene at concentrations up to 0.25 mmol L^{-1}, and limited growth was observed at 0.5 mmol L^{-1}. The results of Van Hamme *et al.* [160] could therefore have been due to the inhibitory effect of benzothiophene at higher concentrations. Decreasing the concentrations of dibenzothiophene and of dibenzothiophene sulfone to 0.01 mmol L^{-1} did not lead to growth with these compounds, supporting the hypothesis that they are not substrates for the desulfurization enzymes produced by JVH1. Significant loss of dibenzothiophene sulfone was observed at all the concentrations tested, but no metabolites were detected and the mechanism of loss (biotic or abiotic) remains unknown. Further studies were undertaken to analyse the activity of JVH1 against two-ring aromatic sulfur compounds (Section 5.3).

Some metabolites were identified arising from compounds used as sulfur sources by JVH1. Oxidized, uncleaved products were observed in extracts of *n*-octyl sulfide cultures (*n*-octyl sulfone), benzyl sulfide cultures (benzyl sulfoxide and benzyl sulfone), and benzyl sulfoxide cultures (benzyl sulfone). PFPP-acid was identified in acidic extracts of PFPS-degrading cultures through comparison of the GC-FID retention time to authentic standards of PFPP-acid and PFPP-OH. PFPP-OH was not detected. Benzoic acid was similarly identified in acidic extracts of benzyl sulfide and benzyl sulfoxide cultures through comparison of the GC-FID retention time to authentic standards of benzoic acid and benzyl alcohol, before and after derivatization with diazomethane.

Table 5.1. Use of organic sulfur compounds by *Rhodococcus* sp. strain JVH1

Compound (incubation time, days)	Initial concentration (mmol S L^{-1})	Growth [‡]	Substrate loss (%) [§]	Metabolites detected
n-Alkyl sulfides				
n-Butyl sulfide (7)	0.1	+	99 ± 1	None found
n-Octyl sulfide (17) [*]	0.05	+	100	***n*-Octyl sulfone**
n-Dodecyl sulfide (7) [*]	0.05	+	78 ± 11	None found
n-Hexadecyl sulfide (7)	0.05	+	54 ± 15	None found
n-Octadecyl sulfide (14) [†]	0.02	ND	100	None found
Aryl terminated sulfides and related compounds				
Benzyl sulfide (8) [*]	0.05	+	100	**Benzyl sulfoxide** **Benzyl sulfone** **Benzoic acid**
Benzyl sulfoxide (8) [*]	0.05	+	72 ± 25	**Benzyl sulfone** **Benzoic acid**
Benzyl sulfone (8) [*]	0.05	+	56 ± 5	None found
PFPS (8) [*]	0.04	+	73 ± 6	**PFPP-acid**
Phenyl sulfide (13) [*]	0.1	-	18 ± 4	None found
Benzyl disulfide (13)	0.1	-	43 ± 15	None found
Cyclic sulfides and related compounds				
1,4-Dithiane (8) [*]	0.05	+	73 ± 2	None found
Tetrahydrothiophene (7)	0.07	+	ND	ND
Tetramethylene sulfoxide (7)	0.07	+	ND	ND
Tetramethylene sulfone (7) [*]	0.04	+	ND	ND
2,4-Dimethylsulfolane (7)	0.07	+	ND	ND
3,4-Dichlorosulfolane (12)	0.05	+	ND	ND
Thiophenic and aromatic sulfur compounds				
Benzothiophene (7) [*]	0.06	+	93 ± 7	None found
Dibenzothiophene (7) [*]	0.05	=	NS	None found
Dibenzothiophene sulfone (7) [*]	0.05	=	70 ± 6	None found
Thianthrene (7) [*]	0.05	=	NS	None found

ND – Not determined; NS – Not significant.

[*] Compounds previously tested by Van Hamme *et al.* [160]

[†] *n*-Octadecyl sulfide dissolved in HMN, see text

[‡] Growth (qualitative OD_{600}) greater than (+), equivalent to (=), or less than (-) sulfur-free negative controls

[§] Relative to sterile controls; average ± standard deviation, n=3

To overcome potential mass transfer limitations from crystalline *n*-octadecyl sulfide to the cells, this substrate was dissolved in HMN as a non-toxic, non-biodegradable organic carrier solvent. The *n*-octadecyl sulfide / HMN solution formed a second liquid phase (1 % by volume) in the culture flasks, providing solubilized *n*-octadecyl sulfide to the culture. This approach approximates the case of whole oil, where large molecules would be solubilized in the bulk oil regardless of their aqueous solubility. Control cultures contained crystalline *n*-octadecyl sulfide as the sole sulfur source. After 2 weeks, all of the initial *n*-octadecyl sulfide was still present in the control cultures, but none was detectable in the cultures in which *n*-octadecyl sulfide was added in HMN. The effect of sulfate was also tested for both crystalline and solubilized *n*-octadecyl sulfide. The presence of sulfate promoted growth in the flask containing crystalline *n*-octadecyl sulfide, resulting in only 63 % recovery of the initial *n*-octadecyl sulfide at the end of incubation. Sulfate did not affect the total degradation of solubilized *n*-octadecyl sulfide. The main pathway for degradation may therefore be attack on the carbon chains, as was identified in the degradation of *n*-octyl sulfide by *R. erythropolis* strain EPWF and *Rhodococcus* sp. strain K1bD (Section 4.4).

5.2.2 Selectivity between thiophenic and sulfidic sulfur compounds

Rhodococcus sp. strain JVH1 was grown with binary combinations of benzothiophene, 1,4-dithiane, and benzyl sulfide as sulfur sources, and the degradation of each compound was compared to degradation as a sole sulfur source (Figure 5.1). Benzothiophene and 1,4-dithiane were degraded at the same rate both as sole sulfur sources and when provided together as sulfur sources. In contrast, the biodegradation of both benzothiophene and of 1,4-dithiane was delayed by the presence of benzyl sulfide in the culture medium. Benzothiophene and 1,4-dithiane did not affect the degradation of benzyl sulfide. Selectivity was also tested using benzothiophene sulfone and benzyl sulfone (Figure 5.2). The rate of degradation of each sulfone was decreased in the presence of the other. These results suggest competitive inhibition between benzyl sulfide and the other two reduced sulfur sources, and between the two sulfones.

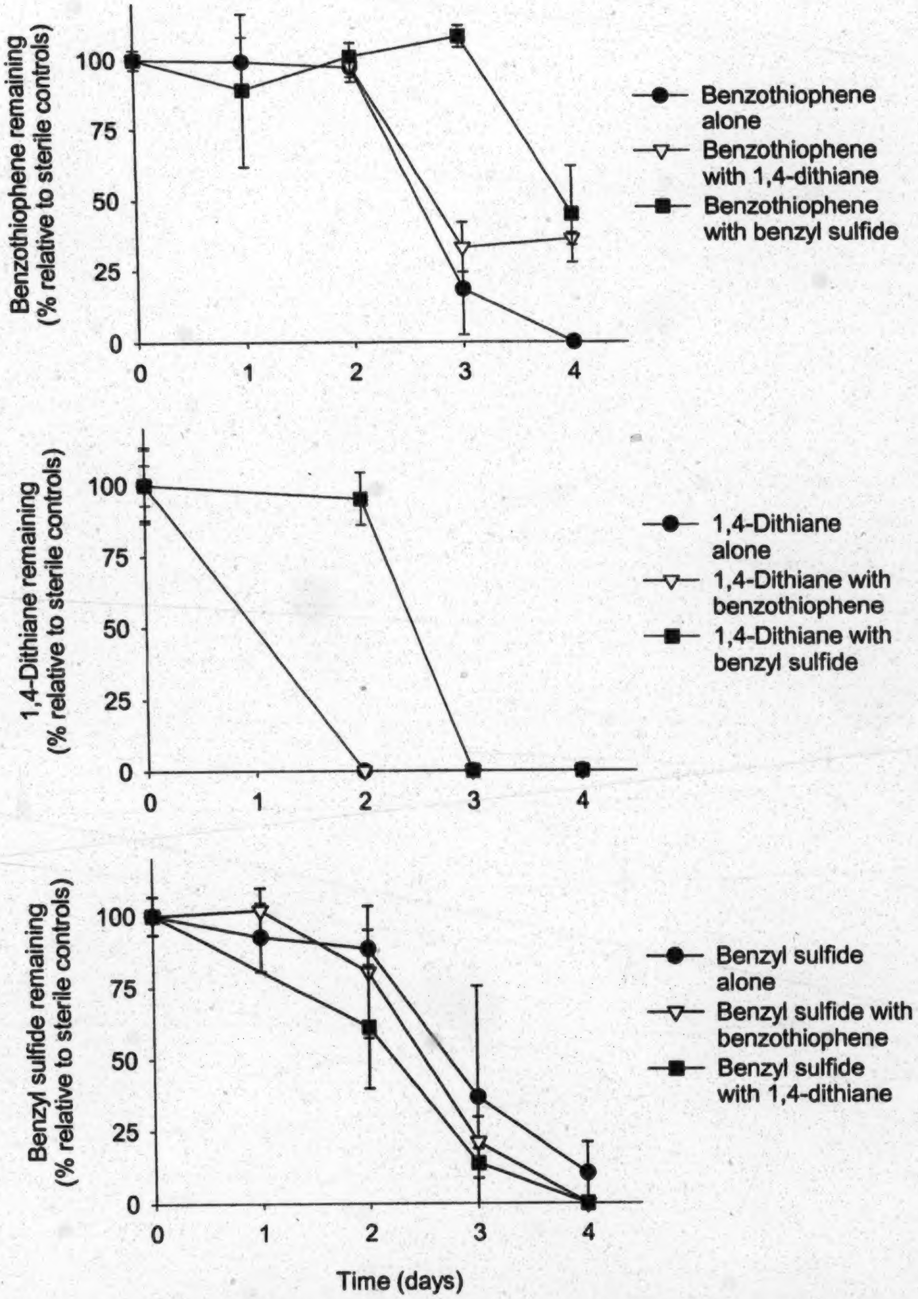


Figure 5.1. **Degradation of benzothiophene, 1,4-dithiane, and benzyl sulfide when provided alone or in binary combinations as sulfur sources for *Rhodococcus* sp. strain JVH1 (average ± standard deviation, n=3). Each compound provided 0.05 mmol S L^{-1} to the culture.**

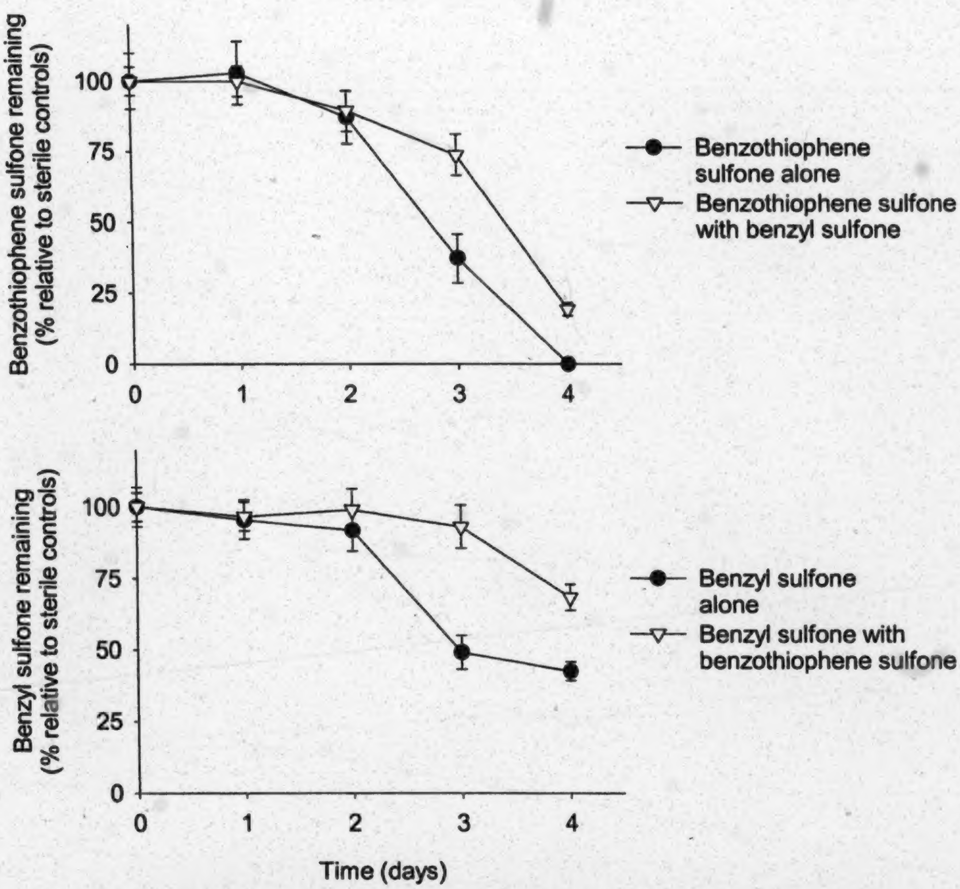


Figure 5.2. Degradation of benzothiophene sulfone and benzyl sulfone when provided alone or together as sulfur sources for *Rhodococcus* sp. strain JVH1 (average ± standard deviation, n=3). Each compound provided 0.05 mmol S L^{-1} to the culture.

5.2.3 Degradation and selectivity in model oils

Selectivity between benzyl sulfide and benzothiophene was further tested in two-liquid-phase cultures, to see if the same patterns were observed when the sulfur compounds were solubilized in a second liquid phase (model oil). This system is expected to have different mass transfer characteristics, changing the availability of the sulfur compounds to the cells and therefore potentially the rate and extent of degradation. The substrates were added in HMN as the carrier phase, giving 0.1 % HMN by volume in the cultures. In the model oil system, the degradation of benzothiophene was not affected by the presence of benzyl sulfide (Figure 5.3). For benzyl sulfide the initial rate was not affected, but the extent of degradation changed in the presence of benzothiophene (Figure 5.3). Benzyl sulfide was fully degraded in 3 days when provided as the sole sulfur source. When benzothiophene was added, only 75 % of the benzyl sulfide was degraded, and this degradation was complete in 2 days. Degradation of benzothiophene was also complete in 2 days.

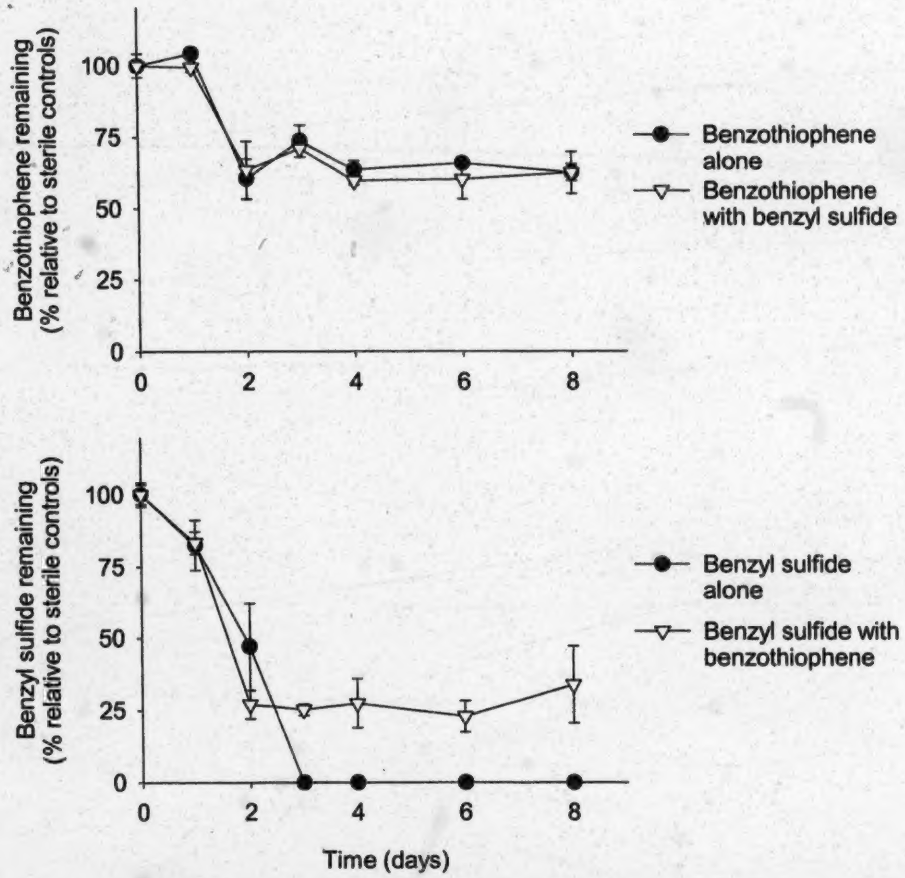


Figure 5.3. **Degradation of benzothiophene and benzyl sulfide when provided alone or together in HMN as sulfur sources for *Rhodococcus* sp. strain JVH1 (average ± standard deviation, n=3). Each compound provided 0.05 mmol S L^{-1} to the culture.**

Visual inspection of the cultures suggested that growth was more rapid when benzothiophene was included as a sulfur source (alone or with benzyl sulfide) than when benzyl sulfide was the sole sulfur source. The increase in biomass could not be measured directly due to adhesion of the cells to the HMN layer. Instead, the concentration of acetate (the carbon source) in the cultures was determined at different times as an indirect estimate of culture growth (Figure 5.4). The consumption of acetate was similar in cultures containing benzothiophene (alone or with benzyl sulfide), whereas an increased lag phase was observed when benzyl sulfide was the sole sulfur source.

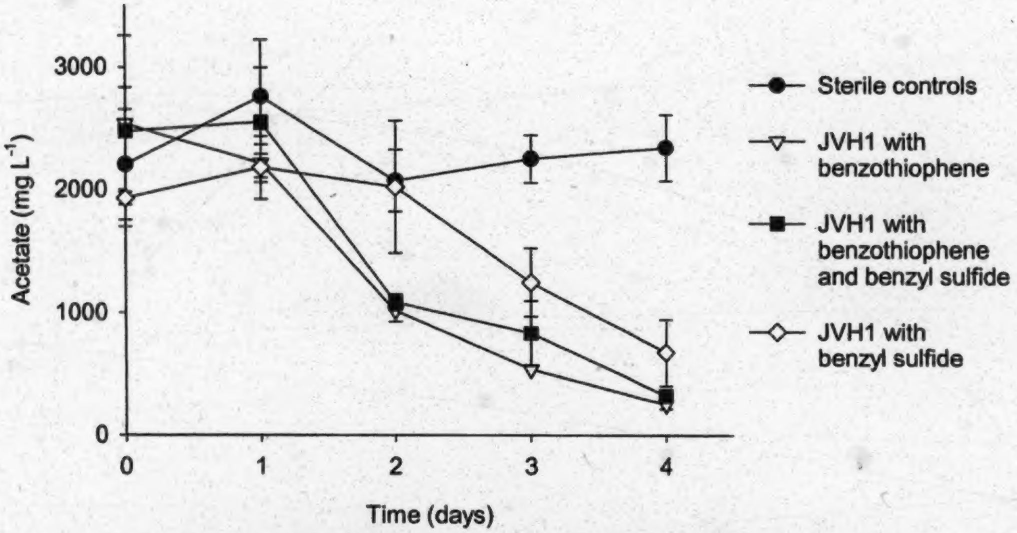


Figure 5.4. Consumption of acetate in cultures of *Rhodococcus* sp. strain JVH1 grown with benzothiophene and benzyl sulfide in HMN as sulfur sources (average ± standard deviation, n=3).

5.3 Desulfurization of benzothiophenes

5.3.1 Effect of sulfate on desulfurization of benzothiophene and benzothiophene sulfone

Bacterial desulfurization of organic sulfur sources is commonly repressed in the presence of inorganic sulfate [142, 159]. The response of desulfurizing cultures to sulfate can therefore be used as an indicator of whether the desulfurization mechanism is sulfur-specific. The effect of sulfate concentration on the use of aliphatic sulfur compounds by

Rhodococcus sp. strain JVH1 is presented in Chapter 7. The effect of sulfate concentration on the degradation of benzothiophene and benzothiophene sulfone by JVH1 was tested to determine whether desulfurization of these thiophenic compounds was also sulfur-specific. Degradation of both compounds was significantly repressed at sulfate concentrations of 0.05 mM or higher (Figure 5.5). The extent of degradation decreased from 100 % with no sulfate to 11 % with 5 mM sulfate for benzothiophene, and from 93 % to 15 % for benzothiophene sulfone. This regulatory effect is evidence that the desulfurization of benzothiophene by JVH1 involves a direct attack at the sulfur atom, as occurs with aliphatic sulfides.

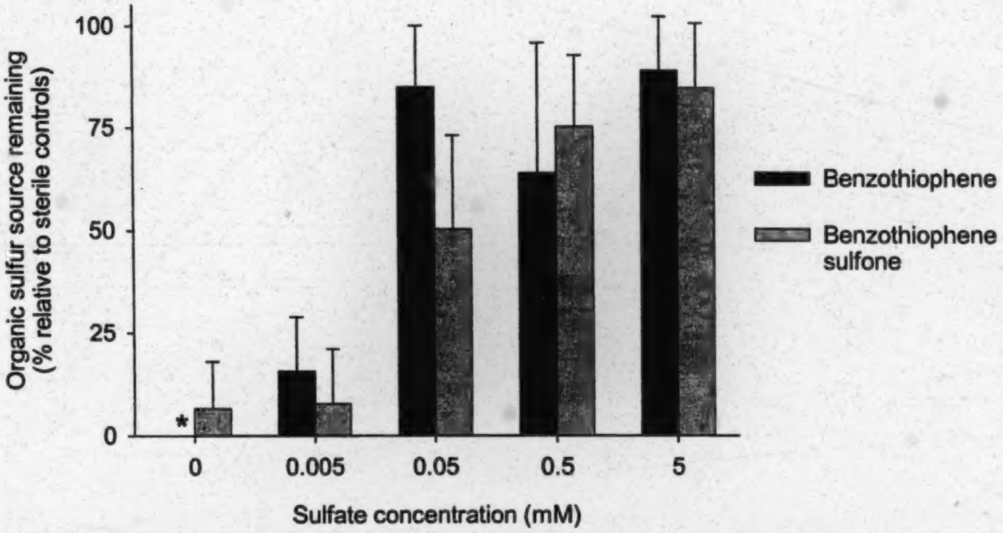


Figure 5.5. Effect of added sulfate on degradation of benzothiophene and benzothiophene sulfone by *Rhodococcus* sp. strain JVH1 after 5 days incubation (average + standard deviation, n=3). Each organic sulfur compound provided 0.05 mmol S L^{-1} to the culture. (* The final benzothiophene concentration was below the detection limit in cultures incubated with benzothiophene and no added sulfate.)

5.3.2 Use of substituted benzothiophenes

To investigate the mechanism of benzothiophene desulfurization, *Rhodococcus* sp. strain JVH1 was incubated with each of the methylbenzothiophene isomers as well as some dimethylbenzothiophene isomers and substituted benzothiophene sulfones provided as the sole sulfur sources for growth. JVH1 was able to grow with all of the monosubstituted benzothiophenes and benzothiophene sulfones at concentrations of approximately 0.1 mmol L^{-1} (Table 5.2). Sensitivity to concentration was observed for some compounds. At 0.15 mmol 4-methylbenzothiophene L^{-1}, growth was suppressed relative to the sulfur-free negative controls, but the cultures did grow with 0.07 mmol 4-methylbenzothiophene L^{-1}. Similarly, JVH1 did not grow with 0.12 mmol 7-ethylbenzothiophene L^{-1}, and only one of three replicate cultures grew with 0.06 mmol 7-ethylbenzothiophene L^{-1}.

Because JVH1 was able to use all of the methylbenzothiophene isomers, it was expected that JVH1 would also be able to use dimethylbenzothiophene isomers as sulfur sources. However, the dimethylbenzothiophene isomers were apparently more inhibitory than the methylbenzothiophene isomers and no growth was observed at the higher concentrations tested (Table 5.3). 4,6-Dimethylbenzothiophene supported growth in all three replicate cultures at 0.04 mmol L^{-1}, while 3,5-dimethylbenzothiophene supported growth at 0.07 mmol L^{-1} in only one of three replicate cultures. Although lower concentrations of the other isomers were not tested due to limitations in measurement, they might be expected to support growth, as long as enough sulfur was still available to the culture.

Table 5.2. Use of unsubstituted and monosubstituted benzothiophenes and benzothiophene sulfones as sole sulfur sources by *Rhodococcus* sp. strain JVH1

Compound [*] (incubation time, days)	Initial concentration (mmol L^{-1})	Growth [†]	Substrate loss [§] (%)
Unsubstituted benzothiophene and benzothiophene sulfone			
Benzothiophene (8)	0.09	+	95 ± 5
(7)	0.05	+	93 ± 1
Benzothiophene sulfone (8)	0.14	+	72 ± 17
Monoalkylbenzothiophenes			
2-MBT (7)	0.11	+	99 ± 1
3-MBT (8)	0.09	+	38 ± 6
4-MBT (11)	0.15	-	33 ± 21
(10)	0.07	+	72 ± 8
5-MBT (7)	0.10	+	87 ± 5
4- & 6-MBT (8)	0.09 total	+	66 ± 3 (4-MBT) 100 (6-MBT)
7-MBT (11)	0.03	+	24 ± 20
7-EBT (11)	0.12	-	NS
(10)	0.06	+ (1/3) [‡] - (2/3) [‡]	64 (1/3) [‡] 15 (2/3) [‡]
Monosubstituted benzothiophene sulfones			
3-MBT sulfone (8)	0.10	+	100
4-MBT sulfone (11)	0.08	+	84 ± 14
5-FBT sulfone (8)	0.10	+	92 ± 13
4- & 6-MBT sulfone (8)	0.08 total	+	86 ± 4 (4-MBT sulfone) 99 ± 2 (6-MBT sulfone)

NS – Not significant

[*] MBT, methylbenzothiophene; EBT, ethylbenzothiophene; FBT, fluorobenzothiophene

[†] Growth (qualitative OD_{600}) greater than (+) or less than (-) sulfur-free negative controls

[‡] If triplicates differed, number in each category is indicated

[§] Relative to sterile controls; average ± standard deviation, n=3, unless otherwise indicated

Table 5.3. **Use of dimethylbenzothiophenes as sole sulfur sources by *Rhodococcus* sp. strain JVH1**

Compound * (incubation time, days)		Initial concentration (mmol L^{-1})	Growth †	Substrate loss § (%)
2,3-DMBT	(11)	0.14	-	NS
	(10)	0.07	-	NS
2,7-DMBT	(11)	0.10	-	NS
	(10)	0.05	-	35 ± 17
3,5-DMBT	(11)	0.14	-	NS
	(10)	0.07	+ (1/3) ‡ - (2/3) ‡	87 (1/3) ‡ NS (2/3) ‡
3,7-DMBT	(11)	0.16	-	NS
	(10)	0.08	-	NS
4,6-DMBT	(11)	0.09	-	9 ± 1
	(7)	0.04	+	89 ± 1

NS – Not significant

* DMBT, dimethylbenzothiophene

† Growth (qualitative OD_{600}) greater than (+) or less than (-) sulfur-free negative controls

‡ If triplicates differed, number in each category is indicated

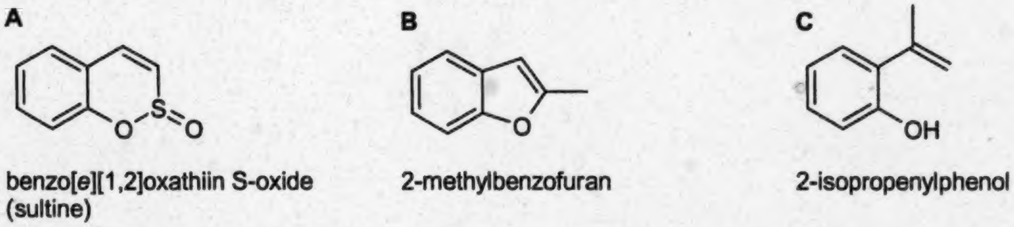
§ Relative to sterile controls; average ± standard deviation, n=3, unless otherwise indicated

5.3.3 Metabolites from desulfurization of benzothiophenes

Culture extracts of *Rhodococcus* sp. strain JVH1 grown with benzothiophene and methylbenzothiophenes were analysed by GC-FID and GC-MS to identify any accumulated metabolites. The proposed metabolites (Table 5.4) are consistent with the published pathways for the desulfurization of benzothiophene and methylbenzothiophenes (Figure 2.4), and include sulfones and sultines (Figure 5.6A). Two different end products were tentatively identified. 2-Methylbenzofuran (Figure 5.6B) was identified in the extract of a culture grown on 2-methylbenzothiophene by comparison of the GC-FID retention time to an authentic standard. In the cultures grown on 3-methylbenzothiophene, the final product may be 2-isopropenylphenol (Figure 5.6C). An authentic standard was not available, but the mass spectrum indicated a molecular weight of 134 and a fragmentation pattern consistent with reported spectra for 2-isopropenylphenol [86] and the isomer 4-isopropenylphenol [62].

Table 5.4. Proposed products formed during the desulfurization of benzothiophene and some methylbenzothiophenes by *Rhodococcus* sp. strain JVH1

Substrate	Proposed products	Evidence
Benzothiophene	Benzothiophene sulfone	Comparison of GC-FID retention time to known standard Comparison of mass spectrum to published spectrum [47]
	Sultine (Figure 5.6A)	Comparison of mass spectrum to published spectrum [47]
2-Methyl benzothiophene	2-Methyl benzothiophene sulfone	Comparison of mass spectrum to library spectrum
	Sultine	Comparison of mass spectrum to published spectrum for sultine formed from benzothiophene [47]
	2-Methylbenzofuran (Figure 5.6B)	Comparison of GC-FID retention time to known standard
3-Methyl benzothiophene	2-Isopropenylphenol (Figure 5.6C)	Molecular weight from mass spectrum Comparison of MS fragmentation to published data for 2-isopropenylphenol [86] and 4-isopropenylphenol [62]
5-Methyl benzothiophene	5-Methyl benzothiophene sulfone	Comparison of mass spectrum to library spectrum for 3-methylbenzothiophene sulfone



A: benzo[e][1,2]oxathiin S-oxide (sultine)

B: 2-methylbenzofuran

C: 2-isopropenylphenol

Figure 5.6. Structures of proposed compounds found in culture extracts of *Rhodococcus* sp. strain JVH1 grown with benzothiophene (A), 2-methylbenzothiophene (B) and 3-methylbenzothiophene (C).

5.4 Alkane degradation

5.4.1 Use of hydrocarbons and heterocyclic aromatic compounds

Rhodococcus sp. strain JVH1 was tested for the ability to degrade a variety of compounds representing different structures found in oil as carbon sources using spray, spread, and vapour plates (Section 3.4.2). JVH1 was able to grow on linear alkanes (*n*-octane, *n*-hexadecane, *n*-eicosane), the isoprenoid alkane pristane, and monoterminally substituted alkanes (*n*-dodecylcyclohexane, 1-phenyldodecane). Growth was not observed on cyclic alkanes (cycloheptane, cyclooctane), the highly branched alkane HMN, or polycyclic aromatic compounds (naphthalene, anthracene, phenanthrene, dibenzothiophene, carbazole), although these compounds were not toxic.

Rhodococcus sp. strain JVH1 may be capable of cometabolic oxidation of hydrocarbons that do not otherwise serve as carbon sources for the culture. JVH1 was added to BHMV medium with HMN alone, HMN with *n*-hexadecane, or *n*-hexadecane alone. After 3 days incubation, the pH of the cultures with *n*-hexadecane (with or without HMN) decreased to 5.5, indicating metabolic activity, and the pH of the cultures with HMN alone stayed at 7. HMN was extracted and quantified, and 43 % loss of HMN was observed in the cultures with *n*-hexadecane, compared to 14 % loss in cultures with HMN alone and no loss in killed-cell controls. Significant transformation of non-growth substrates may, therefore, occur with this culture, in the presence of a suitable primary substrate.

5.4.2 Effect of soluble carbon sources on alkane degradation

Alkane degradation is an undesirable characteristic, because it will lead to biodegradation of the most desirable components of the oil during biological upgrading. The first reaction in *n*-alkane biodegradation is normally terminal hydroxylation to a primary alcohol, catalysed by an alkane hydroxylase enzyme [158]. Transcription of the gene for alkane hydroxylase needs to be repressed in order to block alkane degradation. In some microorganisms, alkane degradation is repressed in the presence of a readily utilized, soluble carbon source such as a sugar or organic acid (catabolite repression) [150].

A time course of *n*-hexadecane degradation by growing cultures of *Rhodococcus* sp. strain JVH1 was measured to determine a suitable time to test for catabolite repression. From a starting concentration of 0.2 % (by volume), 16 % of the *n*-hexadecane was degraded after 1 day, 45 % after 2 days, and 64 % after 3 days (Figure 5.7). Two days was chosen as a suitable time to be able to readily identify significant changes due to catabolite repression or enhanced degradation in the presence of the soluble carbon sources.

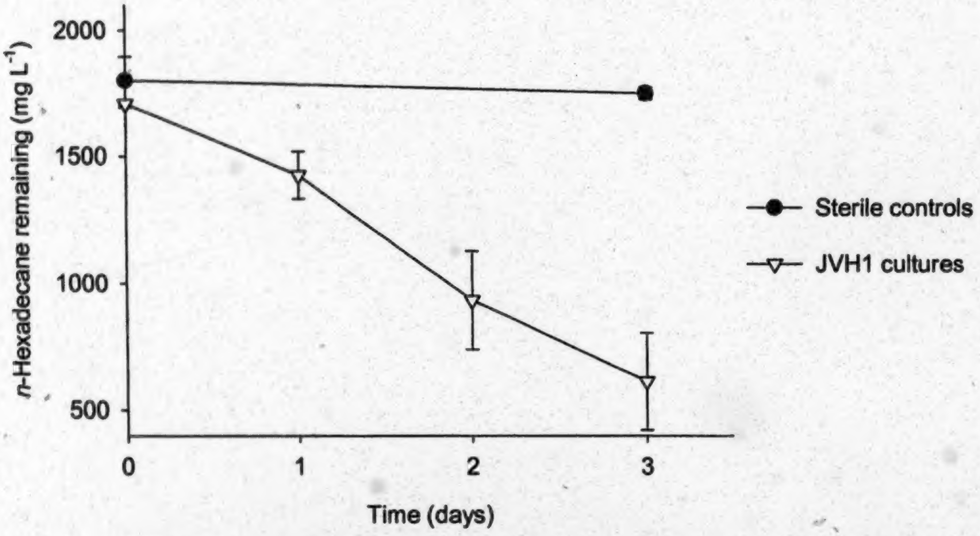


Figure 5.7. Time course of *n*-hexadecane degradation in growing cultures of *Rhodococcus* sp. strain JVH1 (average ± standard deviation, n=3).

The Biolog MicroPlate bacterial identification system (Biolog Inc., Hayward, CA) was used to identify potential catabolites to test as repressors of alkane degradation in *Rhodococcus* sp. strain JVH1. Biolog plates test the ability of an organism to oxidize 95 different substrates, giving a metabolic "fingerprint" that can be used for identification. A variety of sugars, sugar alcohols, and organic acids, all oxidized by JVH1, were selected from the Biolog results. Benzoate was also tested as a known carbon source for JVH1. The concentration of each test substrate was selected to provide 0.01 mol of carbon per litre of medium. The degradation of *n*-hexadecane after two days growth in the presence of each catabolite was measured and compared to degradation after two days growth on *n*-hexadecane alone (Figure 5.8). The amount of *n*-hexadecane degraded was not significantly different from the control with *n*-hexadecane alone for any of the catabolites tested. There was therefore no apparent catabolite repression of alkane degradation in JVH1.

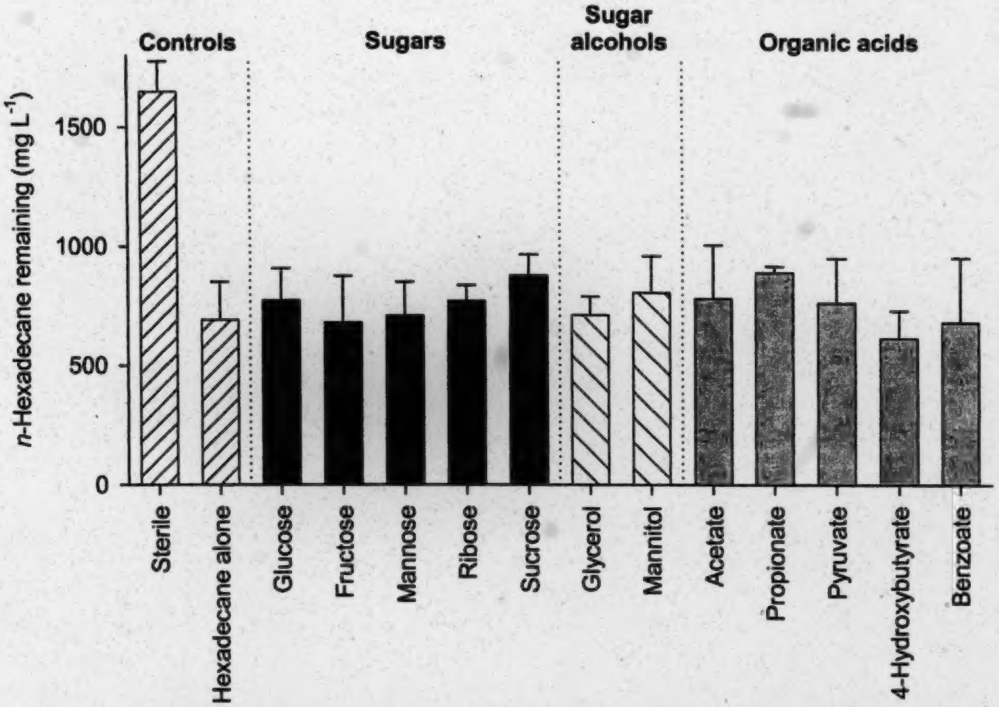


Figure 5.8. Effect of catabolites on *n*-hexadecane degradation after two days in growing cultures of *Rhodococcus* sp. strain JVH1 (average + standard deviation, n=5).

5.5 Discussion and conclusions

The known substrate range of *Rhodococcus* sp. strain JVH1 for aliphatic sulfur compounds was expanded from the work of Van Hamme *et al.* [160], to include shorter and longer dialkyl sulfides (*n*-butyl, *n*-hexadecyl, and *n*-octadecyl sulfides), as well as several cyclic aliphatic sulfur compounds (Table 5.1). Van Hamme *et al.* [160] suggested that JVH1 did not grow under their culture conditions with *n*-octadecyl sulfide as a sulfur source due to the low solubility (and therefore crystalline state) of this substrate in aqueous medium. Degradation of *n*-octadecyl sulfide when dissolved in HMN as a carrier solvent indicated that the limitation previously observed was in fact due to the inability of the organism to utilize the solid substrate, and not due to the size of the individual molecules. The use of a second liquid phase is commonly used to increase the bioavailability of solid compounds with low aqueous solubility (reviewed by Déziel *et al.* [33]). The ability of JVH1 to use *n*-octadecyl sulfide solubilized in HMN is promising for the biological upgrading application where the target molecules (asphaltenes) are very large, but are solubilized in the bulk oil.

Benzyl sulfoxide, benzyl sulfone, and benzoic acid were identified as metabolites produced during growth of *Rhodococcus* sp. strain JVH1 on benzyl sulfide (Table 5.1). These observations were consistent with the desulfurization pathway of JVH1 for PFPS (Figure 2.6) [160], indicating that JVH1 uses a similar mechanism for desulfurization of different aliphatic sulfur compounds. Benzyl alcohol was not found in the JVH1 culture extracts, although it had been found in culture extracts of *Rhodococcus* sp. strain K1bD (Section 4.7). Similarly, only PFPP-acid was found in culture extracts grown on PFPS (Table 5.1), although the alcohol PFPP-OH is a known metabolite [160]. If the oxidation of benzyl alcohol and PFPP-OH to the corresponding carboxylic acids (by JVH1 or abiotically) was rapid, then the intermediate alcohols would not be detected.

The ability of *Rhodococcus* sp. strain JVH1 to desulfurize benzothiophenes contradicted the results of Van Hamme *et al.* [160]. JVH1 was shown to be sensitive to the concentration of benzothiophene and of alkylbenzothiophenes. The published negative result [160] was therefore likely due to the inhibitory effect of benzothiophene at higher concentrations. As previously reported, dibenzothiophene did not serve as a sulfur source for JVH1 [160]. Although many bacteria do use both benzothiophene and dibenzothiophene as sulfur sources [2, 23, 48, 72, 92, 98, 101, 156], it is not uncommon for bacteria capable of desulfurizing benzothiophene to be unable to desulfurize dibenzothiophene (for example, *G. desulfuricans* strain 213E [47] and others [83, 102, 103, 157]), and for bacteria desulfurizing dibenzothiophene to be unable to desulfurize benzothiophene (for example, *R. erythropolis* strain IGTS8 [4] and others [65, 86]). Arensdorf *et al.* [4] showed that a mutation in *dszC*, the gene for dibenzothiophene monooxygenase in *R. erythropolis* strain IGTS8, was required for use of 5-methylbenzothiophene, which was not a substrate for the wild-type enzyme.

Desulfurization of thiophenic sulfur groups would not contribute to a significant reduction in the molecular size of asphaltenes, and is therefore not of use for the proposed biological upgrading application. As with PFPS, the initial attack on benzothiophenes is at the sulfur atom (Section 5.3.3), suggesting that the same enzyme system may be used for both types of compounds. It was therefore of interest to know whether JVH1 exhibits a preference for thiophenic or sulfidic sulfur compounds. For example, *Rhodococcus* sp. strain K1bD showed a preference for the aromatic compound dibenzothiophene over the aliphatic compound 1,4-dithiane as a sulfur source (Figure 4.9).

Although the degradation of benzyl sulfide by *Rhodococcus* sp. strain JVH1 was not affected by the presence of benzothiophene or 1,4-dithiane, degradation of the latter two compounds was delayed in the presence of benzyl sulfide (Figure 5.1). The desulfurization system of JVH1 did, therefore, exhibit some substrate preference for the aliphatic sulfur from benzyl sulfide over the aromatic sulfur in benzothiophene and the aliphatic sulfur in 1,4-dithiane. The lack of selectivity between 1,4-dithiane and benzothiophene showed that the preferences of the microorganism were due to details of chemical structure rather than a simple distinction between aliphatic and aromatic sulfur species.

The degradation rates for both benzyl sulfone and benzothiophene sulfone were reduced when both compounds were added to cultures of *Rhodococcus* sp. strain JVH1 (Figure 5.2). These compounds are intermediates in the desulfurization pathways of benzyl sulfide and benzothiophene, respectively, and would be subject to attack by enzymes catalysing carbon-sulfur bond cleavage rather than sulfur oxidation. Because both compounds were affected, there appears to be a competitive effect on degradation kinetics rather than selectivity at this later stage in the desulfurization pathway.

The addition of benzothiophene and benzyl sulfide dissolved in HMN resulted in decreases in selectivity, lag time, extent of degradation, and time to completion of degradation compared to the single-liquid-phase aqueous system, as summarized in Table 5.5. In biodegradation studies, dissolution of an organic substrate in a second liquid phase is typically used for one of two purposes [33]: to reduce the aqueous phase concentration and thus promote biodegradation of a toxic compound; or to enhance the biodegradation of a compound with low aqueous solubility. In this work, however, the second liquid phase was used to approximate the use of organosulfur species in crude oil, and was therefore not necessarily expected to optimize the bioconversion of the model compounds.

Table 5.5. Summary of differences in the degradation of benzothiophene and benzyl sulfide with and without HMN

	Substrate(s)		No HMN	With HMN
Selectivity			Benzyl sulfide preferred	None observed
Approximate lag time	Benzothiophene	- alone	2 days	All 1 day
		- with benzyl sulfide	3 days	
	Benzyl sulfide	- alone	2 days	
		- with benzothiophene	2 days	
Maximum extent of degradation	Benzothiophene	- alone	All 100 %	50 %
		- with benzyl sulfide		50 %
	Benzyl sulfide	- alone		100 %
		- with benzothiophene		75 %
Time to maximum degradation	Benzothiophene	- alone	3 days	2 days
		- with benzyl sulfide	4 days	2 days
	Benzyl sulfide	- alone	4 days	3 days
		- with benzothiophene	4 days	2 days

The presence of a second liquid phase changes the rate of solubilization or mass transfer of the substrates into the aqueous phase, which can affect the overall degradation rate, and reduces the aqueous phase concentration of the substrates through partitioning, which can affect the rate and extent of degradation. For example, both phenomena were seen to influence the degradation of anthracene by *Sphingomonas* sp. strain BA2 and of pyrene by *Gordonia*-like strain BP9 and *Mycobacterium gilvum* sp. strain VF1, where both the rate and extent of mineralization were decreased when the substrates were dissolved in HMN [113]. For the *Rhodococcus* sp. strain JVH1 cultures, the degradation rates cannot be directly calculated due to the lack of intermediate sampling points. However, in both the aqueous and model oil systems degradation was completed over a period of one or two days, so mass transfer rate limitations do not appear to be a significant challenge to degradation in the model oil system.

Partitioning between the organic and aqueous liquid phases could account for the lack of selectivity, decreased lag time, and decreased extent of degradation in the model oil system. Benzothiophene and benzyl sulfide would be completely dissolved at 0.05 mmol L^{-1} in the aqueous phase in the single-liquid-phase system. The octanol-water partition coefficients (log K_{ow}) are estimated to be 4.33 for benzyl sulfide and 2.99 for benzothiophene [Interactive LogKow (KowWin) Demo, Syracuse Research Corporation; www.syrres.com/esc/est_kowdemo.htm; accessed December 15, 2005]. The concentration of each compound would therefore be approximately 10^3 to 10^4 times lower in the aqueous phase than in the organic (HMN) phase in the two-liquid-phase system. Given a ratio of HMN:water of 1000:1, this partitioning would reduce the aqueous phase concentration by 50 to 90 % compared to the single-liquid-phase system without HMN. The lowered aqueous concentration of benzyl sulfide may be too low to competitively inhibit the degradation of benzothiophene, removing any observable selectivity. Similarly, the lowered aqueous concentrations of benzyl sulfide and benzothiophene could relieve any inhibitory effect of the individual compounds on the growth of the culture, resulting in the reduced lag times. Finally, the lowered aqueous concentration of benzothiophene appears to be close to a minimum threshold for enzymatic activity, accounting for the reduced extent of degradation for benzothiophene in the model oil system.

Partitioning, however, cannot fully explain the extent of conversion of benzyl sulfide in HMN, which reached 100 % when benzyl sulfide was the sole sulfur source, but was limited to 75 % when benzothiophene was also present. The observed differences in culture growth with the different substrate combinations could be related to the differences in benzyl sulfide conversion. Growth of *Rhodococcus* sp. strain JVH1 was indirectly assessed by measuring the acetate concentration in model oil cultures. Acetate was consumed sooner when benzothiophene was present, whereas an increased lag was observed when benzyl sulfide was the sole sulfur source (Figure 5.4). Also, conversion of the sulfur compounds was complete in 2 to 3 days, while acetate was still being consumed by the cultures up to 4 days after inoculation. Although growth phases cannot be clearly distinguished from the acetate data, the continued consumption of acetate indicates that conversion of the sulfur compounds only occurred early in the growth of the cultures. Because growth was more rapid in the cultures with benzothiophene, the active phase for conversion of sulfur compounds started and ended sooner, resulting in incomplete conversion of benzyl sulfide. This effect could also have contributed to the reduced conversion of benzothiophene in the model oil system.

Although the amount of each individual compound converted was decreased in the model oil system, a strong advantage is provided by the absence of the selectivity observed in the aqueous system. Dissolution in an organic phase also allowed attack on *n*-octadecyl sulfide, which was otherwise not used by the organism (Section 5.2.1). In a real oil system, therefore, *Rhodococcus* sp. strain JVH1 would be expected to exhibit a broad activity towards the different sulfur groups present, rather than a specific targeting of certain preferred compounds. Further optimization, for example to determine optimal substrate and biomass concentrations to increase the effective conversion rate during the active growth phase of the culture, would be required to achieve more complete conversion of the material.

Rhodococcus sp. strain JVH1 is the first organism reported to grow on and degrade all six isomers of methylbenzothiophene, as well as some dimethylbenzothiophene isomers, as sole sulfur sources. For example, *R. erythropolis* strain KA2-5-1 could use 3-methylbenzothiophene, 2-ethylbenzothiophene, and 2,7-diethylbenzothiophene, but not benzothiophene, 2-, 5-, or 7-methylbenzothiophene, 7-ethylbenzothiophene, or 5,7-dimethylbenzothiophene [86]; whereas *Gordonia rubropertinctus* strain T08 could use benzothiophene, 2- and 5-methylbenzothiophene, and 2-ethylbenzothiophene, but not 3- or 7-methylbenzothiophene, 7-ethylbenzothiophene, or 2,7- or 5,7-diethylbenzothiophene [102]. The effect of concentration was not tested in these studies [86, 102]. 4- and 6-methylbenzothiophenes are oxidized cometabolically [93, 138], but have never been tested as sulfur sources for bacterial growth.

The ability of *Rhodococcus* sp. strain JVH1 to grow with any of the possible methylbenzothiophene isomers indicated that the attack must be at the sulfur atom, because alkylation would interfere with attack on the ring carbon atoms. The negative effect of sulfate on the use of benzothiophene and benzothiophene sulfone also indicated a sulfur-specific mode of attack. Metabolites identified from benzothiophene and some methylbenzothiophenes were consistent with published pathways for benzothiophene desulfurization (Figure 2.4 [47, 92]), confirming that JVH1 catalyses specific desulfurization of two-ring aromatic sulfur compounds. Products retaining the sulfur atom were sulfones (identified for benzothiophene, 2-methylbenzothiophene, and 5-methylbenzothiophene), and sultines (identified for benzothiophene, Figure 5.6A, and 2-methylbenzothiophene). Sultines are formed from the phenolic sulfinate under acidic extraction conditions (Figure 2.4 [47]). The identification of a sultine is the first direct evidence that a sulfinate is formed from the first carbon-sulfur bond cleavage reaction by JVH1.

Two different desulfurized products were detected in cultures of *Rhodococcus* sp. strain JVH1 grown with methylbenzothiophenes. Extracts of cultures grown on 2-methylbenzothiophene contained 2-methylbenzofuran (Figure 5.6B). This product would be formed by dehydration of 3-(*o*-hydroxyphenyl) propanone under acidic extraction conditions [68]. A phenolic ketone would result if both carbon-sulfur bond cleavage reactions led to oxygenation of the molecule, as occurs in the desulfurization of benzothiophene by *G. desulfuricans* strain 213E (Figure 2.4A) [47]. Extracts of cultures grown on 3-methylbenzothiophene contained 2-isopropenylphenol (Figure 5.6C). In this case, only the first carbon-sulfur bond cleavage oxygenated the molecule, and the desulfination reaction was a hydrolysis, as in the desulfurization of benzothiophene by *Paenibacillus* sp. strain A11-2 (Figure 2.4B) [92]. JVH1 likely possesses both reported pathways for desulfurization of benzothiophenes. Only one other microorganism, *Rhodococcus* sp. strain WU-K2R, has been reported to possess both pathways [83]. Both *o*-hydroxystyrene and benzofuran were detected in extracts of WU-K2R cultures grown with benzothiophene, and both 2′-hydroxynaphthylethene and naphthofuran were detected in extracts of WU-K2R grown with naphtho[2,1-*b*]thiophene [83]. It is unknown at this point whether JVH1 uses different desulfination mechanisms for different compounds, or whether either mechanism could be used for the same compound. JVH1 may also exhibit both pathways in the desulfurization of aliphatic sulfides, although no evidence for a reductive pathway has yet been observed.

Although the position of the methyl group in methylbenzothiophene seemed to affect the toxicity of the isomers, it did not affect the ultimate ability of *Rhodococcus* sp.

strain JVH1 to use these substrates. The inability of JVH1 to use some dimethylbenzothiophene isomers is therefore more likely due to their inhibitory effect than to an enzymatic limitation. The negative effect of the compounds on culture growth is also consistent with inhibition by the alkylbenzothiophenes rather than recalcitrance. Alkylation reduces the aqueous solubility of aromatic compounds, which can be represented by the increase in the octanol-water partition coefficient, K_{ow}. Alkylation is also known to increase the acute toxicity of small aromatic hydrocarbons such as benzene, naphthalene, and phenanthrene [114]. Methylnaphthalenes are degraded more slowly than naphthalene in natural bacterial populations [114], most likely due to the reduced bioavailability of the less soluble alkylated compounds. For benzothiophenes, methylation decreases aqueous solubility and increases toxicity, while oxidation to sulfones or diones increases aqueous solubility and decreases toxicity for both benzothiophene and methylbenzothiophenes [144]. Oxidation of benzothiophenes to their sulfones may therefore be an essential detoxification step for JVH1. Lower aqueous concentrations, due to reduced aqueous solubility, and lower reaction rates, due to mass transfer limitations and possibly steric hindrance, for the more hydrophobic methylbenzothiophenes and dimethylbenzothiophenes, would limit the efficiency of detoxification, and could therefore be responsible for the inability of JVH1 cultures to grow on some of these isomers.

Rhodococcus sp. strain JVH1 used a broad range of compounds with aliphatic sulfur groups as sole sulfur sources for growth, including *n*-alkyl sulfides, alicyclic sulfur compounds, and aryl-terminated linear sulfides. JVH1 could also use benzothiophene, methylbenzothiophenes, and some dimethylbenzothiophenes as sulfur sources. Experimental results indicated that the enzyme system used for desulfurization of PFPS may also be used for desulfurization of other organic sulfur compounds. Metabolites analogous to the PFPS pathway were identified for some compounds: sulfones were formed from *n*-octyl sulfide, benzyl sulfide, and benzothiophenes (analogous to $PFPSO_2$); alcohols were identified from methylbenzothiophenes (analogous to PFPP-OH); and benzoic acid was identified from benzyl sulfide (analogous to PFPP-acid). Also, selectivity was observed between pairs of sulfur compounds, confirming that the same enzyme system was used for more than one substrate. Selectivity was not observed in model oil systems, most likely due to the reduced concentration of substrates in the aqueous phase, resulting from preferential partitioning of the sulfur compounds to the organic (HMN) phase. In a real oil system, therefore, JVH1 would be expected to exhibit broad, nonselective activity towards available sulfur groups within the substrate range of the organism.

6. Activity of *Rhodococcus* sp. strain JVH1 towards Lloydminster heavy crude oil as a sulfur source

6.1 Introduction

To test the activity of *Rhodococcus* sp. strain JVH1 towards crude oil as a sulfur source, Lloydminster crude oil was selected as a representative Alberta heavy crude oil with a high organic sulfur content. This crude oil was expected to have a low inorganic sulfate content, and was filtered before use to remove any entrained mineral solids that could have associated sulfate salts. From 25.13 g of crude oil, 0.11 g of solids was removed by filtration, giving a solids content of 0.44 % by mass in the original crude oil.

Several controls were selected to allow thorough interpretation of the experimental results (Table 3.6). Cell-free sterile controls were included to show changes caused by exposure to and incubation in the aqueous environment as well as in the extraction steps. Killed cell controls were chosen to show any non-catalytic effects of the presence of cells (such as extraction of cellular materials into the oil, or adhesion of oil components to the cells). Spiked controls (sterile and inoculated) contained benzyl sulfide, a known sulfur source for JVH1 [160]. These samples were intended as a positive control, to show whether JVH1 was active towards a known substrate in the presence of the selected crude oil.

Both the original crude oil and the filtered oil were subjected to the same analyses as the extracted oil samples, to allow tracking of any changes caused by the experimental procedures. These analyses included viscosity measurements, sulfur content, and GC-FID/FPD analysis of the saturates and aromatics fractions after partial fractionation.

Raw data, calculations, and statistical analyses for this chapter are shown in Appendix C.

6.2 General changes in the cultures and controls

After incubating for 17 days, the pH was measured for all the cultures and controls. In growing cultures, the pH may drop due to the production of organic acids. The pH was 7 in the live cultures (with and without benzyl sulfide) and in the cell-free sterile controls. Organic acids were therefore not released into the aqueous phase in significant quantities. Although the lack of change in pH may indicate poor growth, it could also result from complete mineralization of the hydrocarbons to carbon dioxide and water or buffering from the medium. Two of the killed controls also had a pH of 7, and the third had a pH of 6.

Before extraction, all the cultures and controls were streaked on PCA to confirm the purity of the live cultures and the sterility of the killed and cell-free sterile controls. All of the live cultures were pure, and the cell-free sterile controls and two of the killed controls were sterile. The third killed control was contaminated, and the plate showed a single colonial morphology distinct from that of *Rhodococcus* sp. strain JVH1. This control had a final pH of 6. The extract from this control was excluded from further analyses.

The extracted oil samples were weighed and the yield calculated to determine gross changes due to the degradation of the oil by the cultures. For the ten extracts, the average yield was 83 % (standard deviation: 1.9 %). Because one of the killed controls was contaminated, triplicate yield measurements were only obtained for the live JVH1 samples (82 % ± 1.2 %) and cell-free sterile controls (84 % ± 0.6 %). Although these values were significantly different at a confidence level of 95 %, the yield was also lower for the two killed controls (83 % and 79 %). The apparent loss of oil in the cultures was therefore likely due to non-biological processes, for example differences in extraction efficiency due to the presence of cells.

6.3 Biotransformation of benzyl sulfide in spiked controls

Test fractionations with benzyl sulfide and benzyl sulfone in crude oil showed that benzyl sulfide eluted with the aromatics fraction and was readily identifiable in the GC-FID/FPD chromatograms for this fraction, while benzyl sulfone did not elute during fractionation and could not therefore be detected. The oil extracts from the spiked controls were partially fractionated and the aromatics fractions analysed by GC-FID/FPD (Sections 3.7.4 and 3.7.5). Significant loss of benzyl sulfide was observed in the live culture compared to the sterile control, with the peak being undetectable in the FID profile (Figure 6.1) and greatly reduced in the FPD profile (Figure 6.2). This result confirmed that cultures of *Rhodococcus* sp. strain JVH1 were active in the presence of heavy crude oil. Because benzyl sulfone is not detectable with the procedures used, the extent of degradation of benzyl sulfide (sulfur oxidation or desulfurization) cannot be assessed.

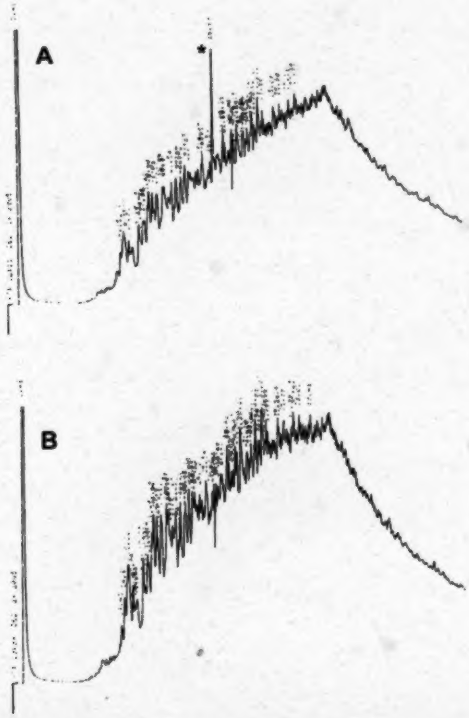


Figure 6.1. FID chromatograms from GC-FID/FPD analysis of the aromatics fraction of spiked control extracts after 17 days incubation: (A) spiked cell-free sterile control (0.2012 g fractionated); (B) spiked JVH1 live test culture (0.2128 g). (* – benzyl sulfide)

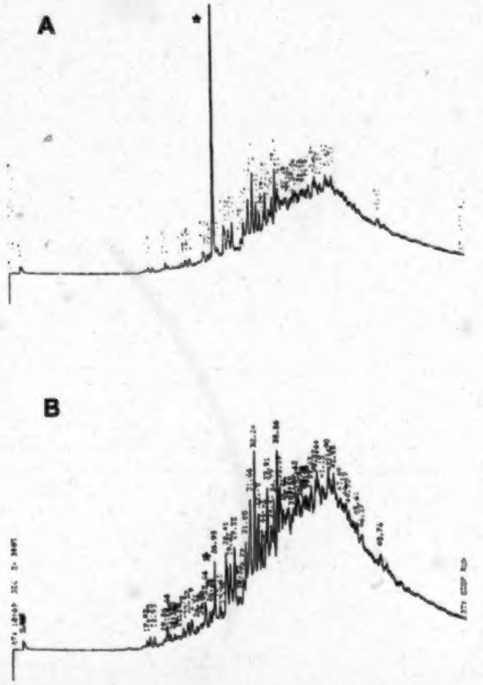


Figure 6.2. FPD chromatograms from GC-FID/FPD analysis of the aromatics fraction of spiked control extracts after 17 days incubation: (A) spiked cell-free sterile control (0.2012 g fractionated); (B) spiked JVH1 live test culture (0.2128 g). (* – benzyl sulfide)

6.4 Viscosity

The dynamic viscosity of the crude and filtered oils and of the pooled extracted oil samples was measured over a range of shear rates from 0.1 s^{-1} to 100 s^{-1}. Representative results for the filtered oil are shown in Figure 6.3. Crude oil samples are expected to behave as Newtonian fluids, exhibiting a constant dynamic viscosity independent of shear rate. For all the samples, however, there was some variability in the apparent viscosity at shear rates below 1 s^{-1}, as well as a decrease in the apparent viscosity at shear rates above 10 s^{-1}. The decrease at higher shear rates may be due to viscous heating of the sample. For each sample and temperature, therefore, the ten viscosity measurements (five per run) obtained at shear rates from 1 s^{-1} to 10 s^{-1} were used to obtain an average value for the sample and to perform statistical comparisons.

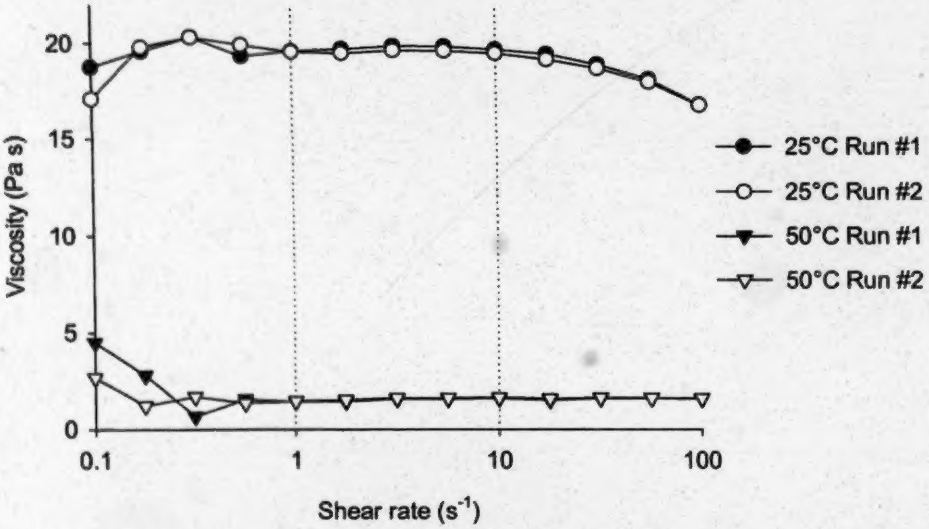


Figure 6.3. Representative plot of the viscosity measurements as a function of shear rate for the filtered oil. Reference lines bound the range of shear rates used for further analyses.

The viscosities obtained for each of the five samples are given in Table 6.1. The viscosity was much higher in the filtered oil than in the crude oil, and also much higher in the extracts than in the filtered oil, at both 25°C and at 50°C. Among the extracts, the sterile control had the lowest viscosity and the live culture extract had the highest viscosity at both temperatures.

Statistical comparisons were performed to determine whether differences among the extract viscosities were significant, using a significance level of 95 %. Single-factor analysis of variance (ANOVA) [109] was used to first determine whether any of the samples were significantly different. This type of ANOVA uses the *F*-statistic to test for differences among the three samples, and provides an estimate of experimental variability (the mean square error of the residuals), which can be used for further statistical analysis. At both temperatures highly significant differences were indicated between the samples ($P = 7.6\times10^{-18}$ at 25°C ; $P = 5.8\times10^{-15}$ at 50°C).

Table 6.1. **Dynamic viscosity and sulfur content of the crude and filtered oils and of the extracted oil samples after 17 days incubation**

	Viscosity (Pa s) *		Sulfur (weight %) †
	25°C	50°C	
Crude oil	1.60 ± 0.095	0.322 ± 0.0463	2.88 ± 0.021
Filtered oil	19.6 ± 0.13	1.54 ± 0.078	3.23 ± 0.024
Sterile controls	51.6 ± 1.48	3.20 ± 0.085	3.38 ± 0.014
Live cultures	78.5 ± 2.62	3.84 ± 0.104	3.46 ± 0.012
Killed controls	69.6 ± 3.83	3.42 ± 0.072	3.38 ± 0.015

* Average ± standard deviation, n=10; pooled extracts analysed

† Weighted average ± standard deviation; individual extracts analysed

Tukey's method for multiple comparisons [109] was used to identify which samples had significantly different viscosities at each temperature. Tukey's test is more conservative than multiple *t*-tests, taking into account that for a large number of paired comparisons there is an increased probability that at least one of the results will be wrong. Instead, the Studentized range statistic, *q*, is used to calculate the "Honestly Significant Difference" (HSD) for two samples at a given confidence level, using the mean square error of the residuals from the ANOVA. The HSDs and the actual differences are compared in Table 6.2. The viscosities of all the samples were found to be significantly different from each other at both 25°C and at 50°C, at a confidence level of 95 %. The presence of cells (live or killed) resulted in an increase in the viscosity of the extracted oil over the cell-free sterile controls, and the presence of live, metabolically active cells resulted in a further increase in oil viscosity over the killed cell controls.

Table 6.2. Results of Tukey's test used to make multiple paired comparisons of the pooled extract viscosities. An absolute difference greater than the HSD is significant at a 95 % confidence level

	Absolute difference (Pa s)		Significant?	
	Killed controls	Sterile controls	Killed controls	Sterile controls
25°C (HSD = 3.12 Pa s)				
Live cultures	8.91	26.9	Yes	Yes
Killed controls		18.0		Yes
50°C (HSD = 0.0975 Pa s)				
Live cultures	0.424	0.646	Yes	Yes
Killed controls		0.223		Yes

6.5 Saturates and aromatics profiles

The FID chromatograms obtained by GC-FID/FPD for the saturates fractions are shown in Figure 6.4 and Figure 6.5. Loss of lighter compounds, which elute earlier in the chromatogram, is evident in the filtered oil (Figure 6.4B) compared to the crude oil (Figure 6.4A), and in the extracts (Figure 6.5A-C) compared to the filtered oil. These losses are expected evaporative losses due to the various solvent removal steps during preparation and analysis. Some of these losses are reflected in the yields of the extracted oil samples, which were all close to 80 % (Section 6.2). Solvent was removed from the filtered oil once, through evaporation at atmospheric temperature and pressure. For the extracts, however, solvent was removed several times before fractionation: evaporation at atmospheric temperature and pressure after filtration and dispensing; rotary evaporation at reduced pressure and increased temperature followed by atmospheric evaporation after extraction; and atmospheric evaporation after both sulfur analysis and viscosity measurements. Some losses could also occur during aerobic incubation of the flasks, because the flasks were closed with foam stoppers and not sealed. The extracts were therefore expected to show greater abiotic mass losses than the filtered oil, as a result of these multiple processing steps.

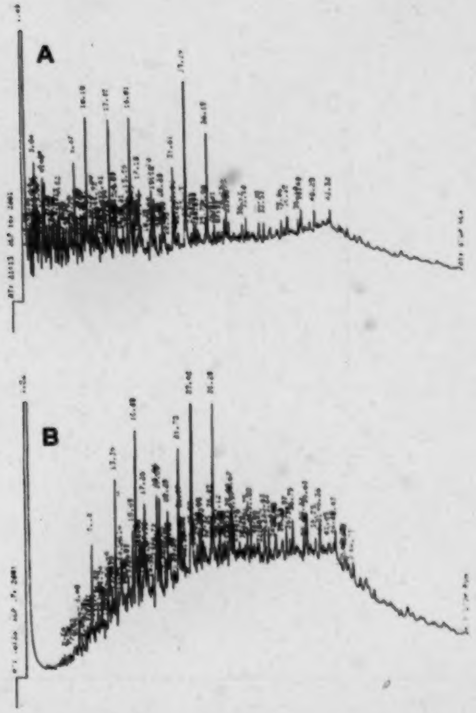


Figure 6.4. FID chromatograms from GC-FID/FPD analysis of the saturates fraction of the crude and filtered oils: (A) crude oil (0.2136 g fractionated); (B) filtered oil (0.2001 g).

The isoprenoid alkanes pristane and phytane are readily identified in the pooled sterile control extract, as indicated in Figure 6.5A. Large *n*-alkanes (from *n*-pentadecane up to *n*-pentacosane) can also be resolved (Figure 6.5A). The *n*-alkanes cannot be distinguished in the pooled live culture extract (Figure 6.5B), but still remain in the pooled killed control extract (Figure 6.5C). The removal of the *n*-alkanes was therefore due to the activity of the live *Rhodococcus* sp. strain JVH1 culture. In addition to *n*-alkanes, JVH1 is also known to degrade pristane (Section 5.4.1). The amount of pristane may be reduced in the live culture extract compared to the cell-free and killed-cell controls; however, loss of pristane cannot be assessed quantitatively without an internal standard. The FPD chromatograms showed that there were no sulfur-containing species in the saturates fraction of the crude oil, so the transformation of sulfur species cannot be assessed from this fraction.

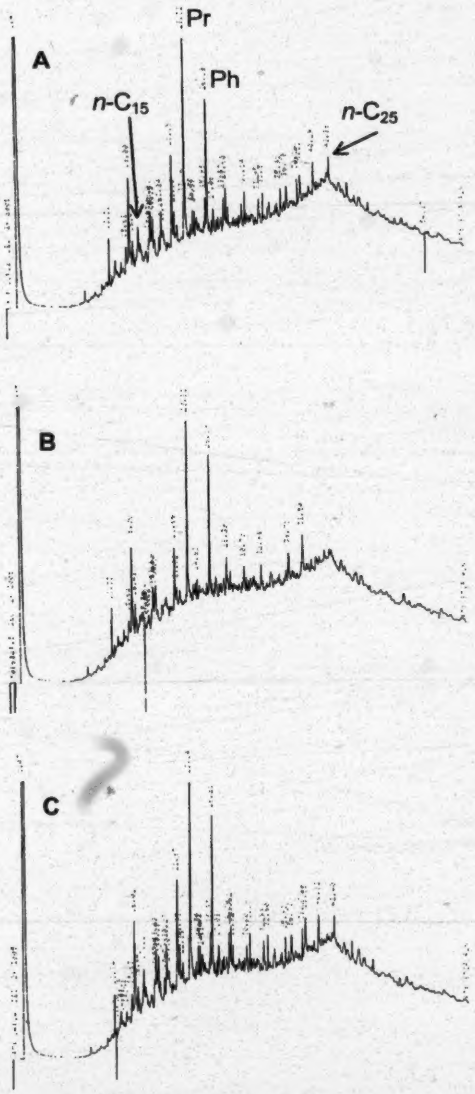


Figure 6.5. FID chromatograms from GC-FID/FPD analysis of the saturates fraction of the pooled extracted oil samples after 17 days incubation: (A) cell-free sterile controls (0.2060 g fractionated); (B) JVH1 live test cultures (0.2125 g); (C) killed controls (0.2015 g). (Pr – pristane; Ph – phytane; *n*-C_{15} – *n*-pentadecane; *n*-C_{25} – *n*-pentacosane)

The FID chromatograms for the aromatics fractions also showed a loss of lighter compounds in the filtered oil compared to the crude oil and in the extracts compared to the filtered oil (not shown). No other differences were apparent in the profiles of the extracts. Sulfur species (larger than benzothiophene) were evident in the FPD chromatograms for the aromatics fractions, and no differences were observed among the extracted samples (not shown). This observation was expected because although JVH1 does desulfurize benzothiophene and methylbenzothiophenes (Section 5.3), JVH1 is not active towards dibenzothiophene and would presumably also not attack alkyldibenzothiophenes or larger thiophenic compounds.

6.6 Sulfur content

The total sulfur content of the crude and filtered oils and of each of the extracted oil samples was measured to see whether any desulfurization of the oil could be detected. The sulfur content of the filtered oil and of the extracts was higher than in the crude oil (Table 6.1). The observed increase in the sulfur content is most likely due to the evaporative losses of light hydrocarbons, observed in the FID chromatograms, leading to a relative enrichment in heavier heteroatomic species in the oil. The sulfur content of the live culture extracts was also higher than both the cell-free sterile controls and the killed-cell controls.

The sulfur content of the three groups of extracts was compared using a single-factor ANOVA for comparison of multiple samples of different sizes. The ANOVA indicated that significant differences existed among the samples at a confidence level of 95 % ($P = 0.044$). The Tukey-Kramer test was used to try to identify which pairs were significantly different. This test is equivalent to Tukey's test, but uses the geometric mean to account for differences in samples sizes. The HSDs and the actual differences are compared in Table 6.3. None of the pairs of samples were found to be significantly different, using a confidence level of 95 %. However, the HSD between the live cultures and the sterile controls was very close to the actual difference, and the P-value for the ANOVA was very close to the significance level, so these two samples may in fact be different. However, since the sulfur content of the live cultures was higher than the sterile controls, the cultures did not carry out significant desulfurization of the oil.

Table 6.3. **Results of the Tukey-Kramer test used to make multiple paired comparisons of the individual extract sulfur contents. An absolute difference greater than the HSD is significant at a 95 % confidence level**

	HSD (weight %)		Absolute difference (weight %)		Significant?	
	Killed controls	Sterile controls	Killed	Sterile	Killed	Sterile
Live cultures	0.0948	0.0848	0.0755	0.0845	**No**	**No**
Killed controls		0.0948		0.0090		**No**

6.7 Discussion and conclusions

Rhodococcus sp. strain JVH1 was metabolically active towards filtered Lloydminster heavy crude oil provided as the sole sulfur source (glycerol was provided as a carbon source), resulting in the removal of *n*-alkanes from the oil, as well as the removal of benzyl sulfide in a spiked control. No decrease in sulfur content was detected. This result may be due to the low sulfur requirements of the bacterium (which grows with as little as 0.005 mmol of added S per litre of medium) compared to the amount of sulfur introduced in the oil (the filtered oil contained 2.88 % S by weight, resulting in a concentration of 4.5 mmol S per litre of medium). Kaufman *et al.* [70] similarly reported negligible decreases in the total sulfur content of crude oils after biodesulfurization by the dibenzothiophene-desulfurizing bacterium *R. erythropolis* strain IGTS8, even though the culture removed 98 % of the dibenzothiophene present and over 90 % of most of the alkylated dibenzothiophenes quantified.

Rhodococccus sp. strain JVH1 is known to use *n*-alkanes and pristane as carbon sources, and to cometabolize the highly branched alkane HMN (Section 5.4.1). Although JVH1 removed the *n*-alkanes from crude oil, little or no activity was observed towards the isoprenoid alkanes pristane and phytane (Figure 6.5). Peaks representing other components of the saturates fraction, most likely branched and cyclic alkanes, also remained in the GC-FID profile of the live culture extract (Figure 6.5). Competition experiments with radiolabelled substrates showed that although *n*-hexadecane is mineralized in the presence of pristane or *n*-octyl sulfide, mineralization of pristane is strongly inhibited or even eliminated in the presence of *n*-hexadecane or *n*-octyl sulfide [*]. The same phenomenon may therefore have occurred for the whole oil, with preferential degradation of the *n*-alkanes preventing activity towards the branched and cyclic species.

The viscosity of the extracted oil was higher after exposure to live cultures of *Rhodococcus* sp. strain JVH1, contrary to the desired result of viscosity reduction. This increase may have been partly due to the presence of cells, and not to biologically catalysed changes in the oil, since the viscosity of the killed control extract was also significantly higher than the sterile control extract. This trend could be the result of extraction of cellular material into the oil, which increased the viscosity of the mixed sample. Alternately, the extraction efficiency of the different oil components could have been altered due to selective interaction of certain species from the oil with the cells.

The viscosity of the live culture extract was also significantly higher than the killed control, indicating that some of the increase was caused by the metabolic activity of the live cells. The removal of the *n*-alkanes, observed in the saturates profile, could account for some of this increase. Oxidation of the oil to produce more polar species, including carbonyl, alcohol, and sulfoxide functionalities, could also contribute to an increase in viscosity. Fourier-transform infrared analysis would be useful to assess whether oxidized species were introduced through the activity of the live cultures.

Further work is required to improve the selectivity of the biocatalyst, in order to increase the level of conversion of the aliphatic sulfides and minimize the oxidation and removal of the valuable hydrocarbon material. Without much higher levels of conversion of the sulfur in the oil, the biocatalyst could not substantially reduce the viscosity of the crude oil.

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[*] Wloka, M., and J.M. Foght. 2005. Hydrocarbon accumulation and alkyl-sulfide degradation by *Rhodococcus* sp. strain JVH1. Unpublished report, Department of Biological Sciences, University of Alberta. April 29, 55 pp.

7. Regulation of desulfurization by sulfate in *Rhodococcus* sp. strain JVH1

7.1 Introduction

A common feature of bacteria able to use organic sulfur sources is repression of desulfurization activity in the presence of sulfate [142, 159]. Sulfate exhibited a regulatory effect on the use of aliphatic sulfides by isolates from enrichment cultures (Figure 4.7, Figure 4.8), and on the use of benzothiophene by *Rhodococcus* sp. strain JVH1 (Figure 5.5). In this chapter, further screening experiments are presented showing that the use of aliphatic sulfides by JVH1 is also regulated by sulfate, and that the regulatory effect is most likely repression. For field treatment of heavy oil, sulfate repression in JVH1 must be avoided, because inorganic sulfate could be present in the produced water. Experiments were conducted to investigate naturally occurring biological mechanisms to alleviate sulfate repression, without requiring genetic manipulation of the organism.

Reduction of intracellular sulfate concentration may be sufficient to stop sulfate repression. This effect could potentially be accomplished by either increasing sulfur demand, or by decreasing the transport and/or metabolism of sulfate. In many microorganisms, thiols (such as glutathione and mycothiol) are produced in response to environmental conditions such as osmotic stress [115, 148]. Increased production of thiols increases sulfur demand [38], and may therefore reduce the intracellular sulfate concentration. In order to test this hypothesis, the effect of salt concentration on sulfate repression was tested.

There is evidence that metabolites of sulfate, not sulfate itself, are the signalling molecules causing repression of alternate sulfur metabolism pathways [22, 142]. Inhibition of sulfate transport or metabolism should therefore eliminate sulfate repression. Sulfate analogues may compete for sulfate transport systems [116, 117, 119] and sulfate metabolic enzymes, and reduce the amount of sulfate that enters the cell and is transformed to repressive metabolites. In addition, if sulfate transport or metabolism was impaired by the presence of sulfate analogues, the cells would be dependent on alternate sulfur sources to support growth. Four sulfate analogues, selenate, chromate, molybdate, and tungstate, were tested for their effect on sulfate repression in *Rhodococcus* sp. strain JVH1.

7.2 Effect of sulfate concentration on use of aliphatic sulfur compounds

7.2.1 Growing cultures

The response of *Rhodococcus* sp. strain JVH1 to sulfate was explored by testing the effect of sulfate concentration on the use of PFPS, 1,4-dithiane, benzyl sulfide, benzyl sulfoxide, benzyl sulfone, and *n*-octyl sulfide in the presence of acetate as carbon source. At the lowest sulfate concentrations (0 and 0.005 mmol L^{-1}), comparable amounts of PFPS remained in the cultures and no sulfone ($PFPSO_2$) was detected (Figure 7.1A). At 0.05 mmol sulfate L^{-1}, however, $PFPSO_2$ was detected, and the total amount of non-cleaved compounds (PFPS and $PFPSO_2$) increased, indicating that this concentration was sufficient to exert a regulatory effect on the metabolism of this compound. Metabolism of 1,4-dithiane was also decreased in the presence of sulfate (Figure 7.1B).

The effect of sulfate concentration on each of the sulfur oxidation and carbon-sulfur bond cleavage steps of desulfurization was tested using benzyl sulfide, sulfoxide, and sulfone. Disappearance of benzyl sulfide and of benzyl sulfoxide was not significantly affected by sulfate (Figure 7.2A,B), but in both cases the uncleaved metabolite benzyl sulfone accumulated to a greater extent at higher sulfate concentrations. (Recovery of benzyl sulfoxide in the sterile controls was low due to low purity and poor extraction efficiency of this compound.) Benzyl sulfone accumulation also increased at the upper sulfate concentrations when this compound was provided as the initial substrate (Figure 7.2C).

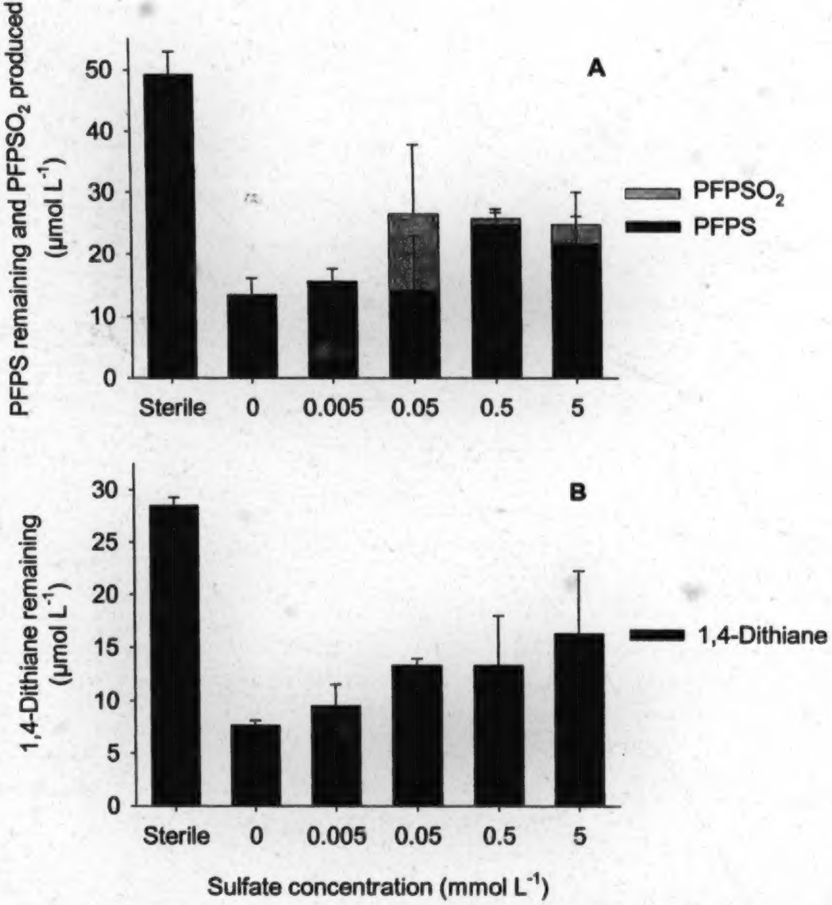

Figure 7.1. **Effect of sulfate concentration on biotransformation by *Rhodococcus* sp. strain JVH1 of (A) PFPS and (B) 1,4-dithiane after 8 days incubation (average + standard deviation, n=3).**

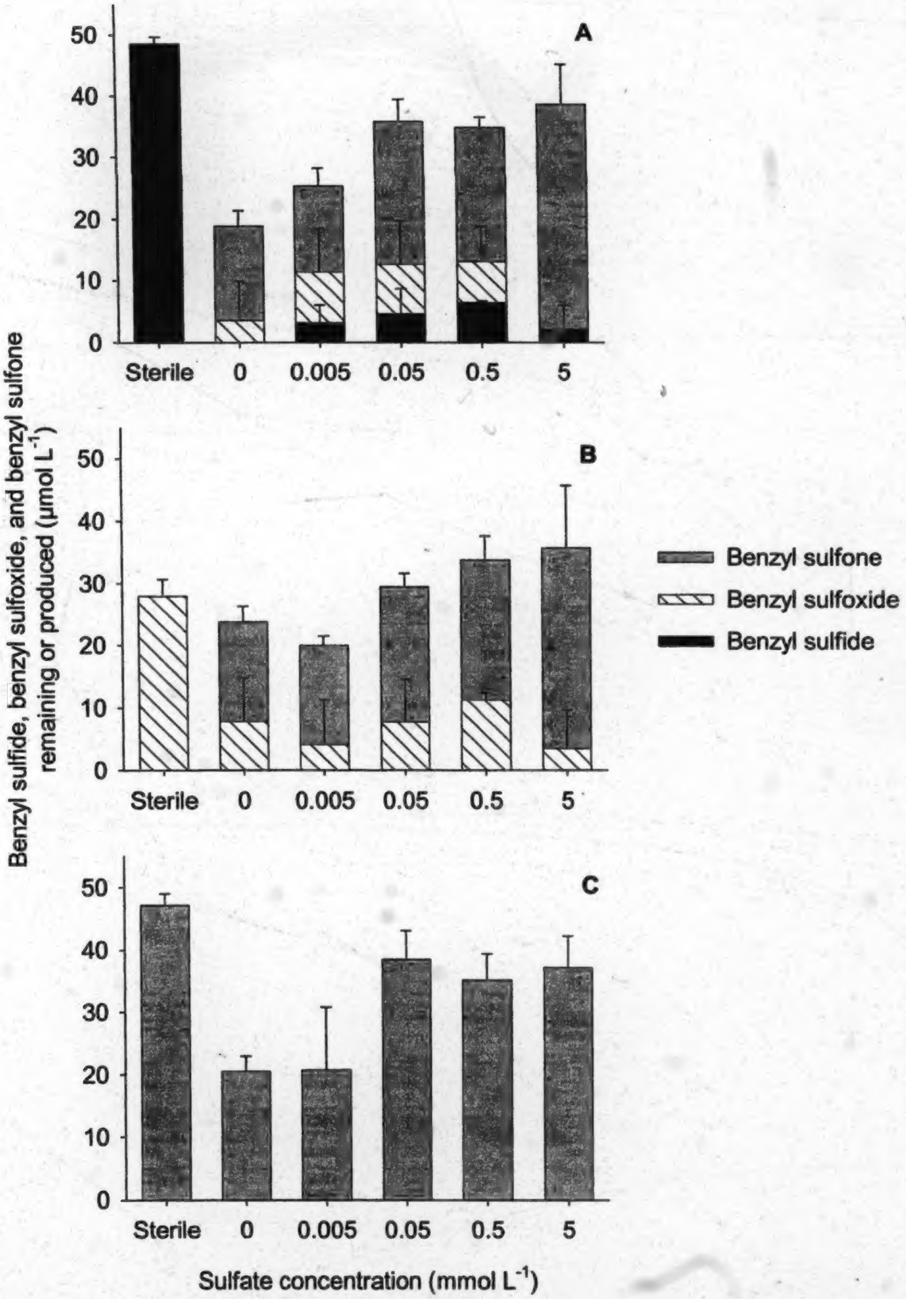

Figure 7.2. **Effect of sulfate concentration on biotransformation by *Rhodococcus* sp. strain JVH1 of (A) benzyl sulfide, (B) benzyl sulfoxide, and (C) benzyl sulfone after 8 days incubation (average + standard deviation, n=3).**

Only traces of remaining *n*-octyl sulfide were found after incubation with JVH1 at all sulfate concentrations (Figure 7.3A). Not all of these cultures grew, however, and various amounts of octyl sulfone were observed in the culture extracts. Octyl sulfone was not quantified because an authentic standard was not available, so the relative accumulation was calculated with respect to the internal standard, thianthrene (Figure 7.3B). This calculation showed a general trend towards accumulation of octyl sulfone in cultures at low sulfate concentrations that did not grow, compared to those that did grow. These cultures were, therefore, sulfur-limited, because the liberation of the sulfur from the sulfone coincided with the growth of the cultures. Octyl sulfone also accumulated in all of the cultures at higher sulfate concentrations (Figure 7.3B). This result is consistent with the observed accumulation of benzyl sulfone in cultures grown with benzyl sulfide or benzyl sulfoxide.

7.2.2 Resting cell cultures

In other bacteria, the regulatory mechanism of sulfate is repression, where sulfate prevents transcription of the genes encoding the enzymes involved in use of organic sulfur sources [142, 159]. JVH1 was grown with 1,4-dithiane or sulfate as the sulfur source, then washed and suspended in phosphate buffer to achieve resting cell suspensions. Portions of the suspensions were autoclaved to provide killed cell controls. The live and killed cell suspensions were then incubated for 3 days with benzyl sulfone with and without sulfate. Benzyl sulfone was selected as the test compound because desulfurization of this compound showed clear discrimination between the effects of high and low sulfate concentrations (Figure 7.2C). Significant loss of benzyl sulfone compared to the cell-free sterile controls was only observed in the live cell suspensions grown on 1,4-dithiane (Figure 7.4). Expression of the desulfurization enzymes was therefore repressed during pre-growth on sulfate. There was no difference in the loss of benzyl sulfone observed with or without sulfate added to the buffer. The desulfurization enzymes were therefore not inhibited by sulfate. The regulatory effect of sulfate is therefore most likely only due to repression.

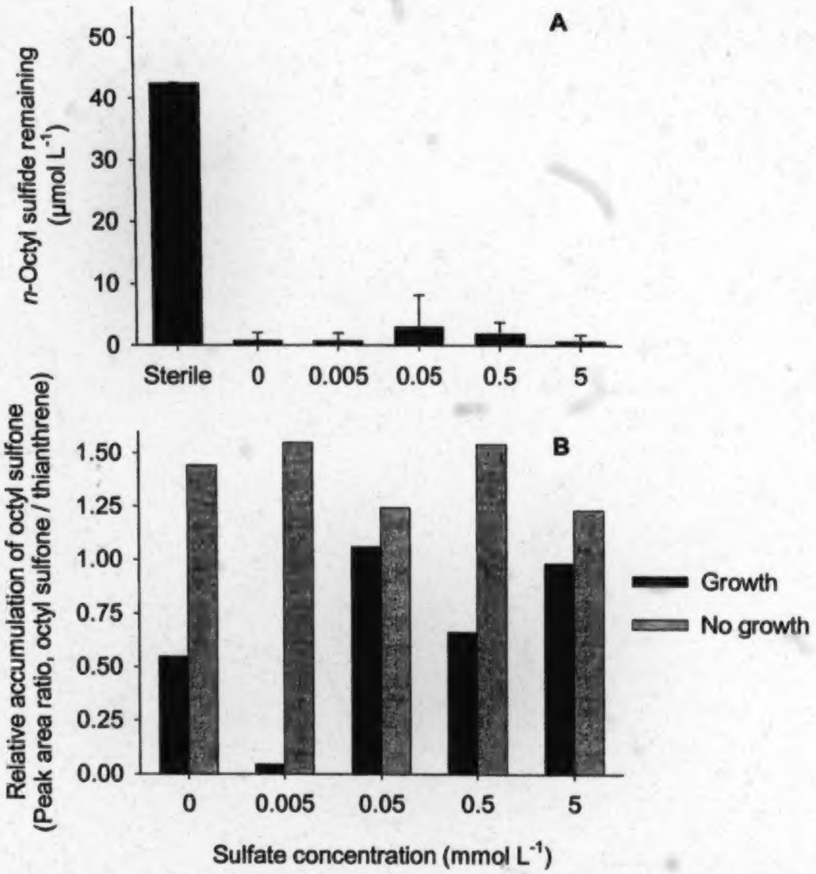

Figure 7.3. **Effect of sulfate concentration on biotransformation of *n*-octyl sulfide by *Rhodococcus* sp. strain JVH1 after 14 days incubation: (A) amount of *n*-octyl sulfide transformed at different sulfate concentrations (average + standard deviation, n=3); (B) relative accumulation of octyl sulfone at different sulfate concentrations in cultures that did or did not grow, expressed with respect to the internal standard (n=3 total at each concentration).**

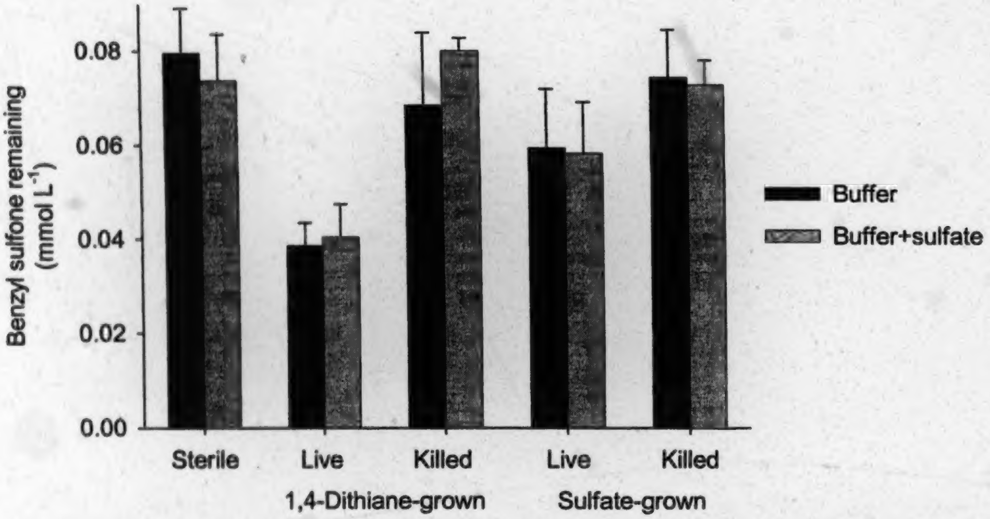

Figure 7.4. Degradation of benzyl sulfone in resting and killed cell suspensions of *Rhodococcus* sp. strain JVH1 after 3 days incubation with and without sulfate in the suspension buffer (average + standard deviation, n=3).

7.3 Attempts to alleviate the effect of sulfate repression on desulfurization

7.3.1 Effect of high salt concentration on sulfate repression

The salt tolerance of *Rhodococcus* sp. strain JVH1 was tested in TSB, a rich medium that promotes rapid growth of this organism. Salt (NaCl) concentrations from 5 to 150 g L^{-1} were used. As shown in Figure 7.5A, the growth rate decreased and the lag period increased with increasing salt concentrations. JVH1 was able to grow in TSB with up to 75 g NaCl L^{-1}. In a defined medium (SFMM+4Ac containing 5 mmol sulfate L^{-1}), JVH1 grew with up to 50 g NaCl L^{-1} (Figure 7.5B).

The effect of sulfate on desulfurization of benzyl sulfone was tested with 1, 25, 50, and 75 g NaCl L^{-1} added to the medium. These salt concentrations were selected to span the range of salt tolerance of JVH1. At 1 g NaCl L^{-1}, the typical effects of sulfate repression were observed (Figure 7.6), with significant loss of benzyl sulfone occurring only at the lowest sulfate concentrations. At the higher salt concentrations, however, although there was growth in the cultures, little or no loss of benzyl sulfone was measured with or without added sulfate (Figure 7.6).

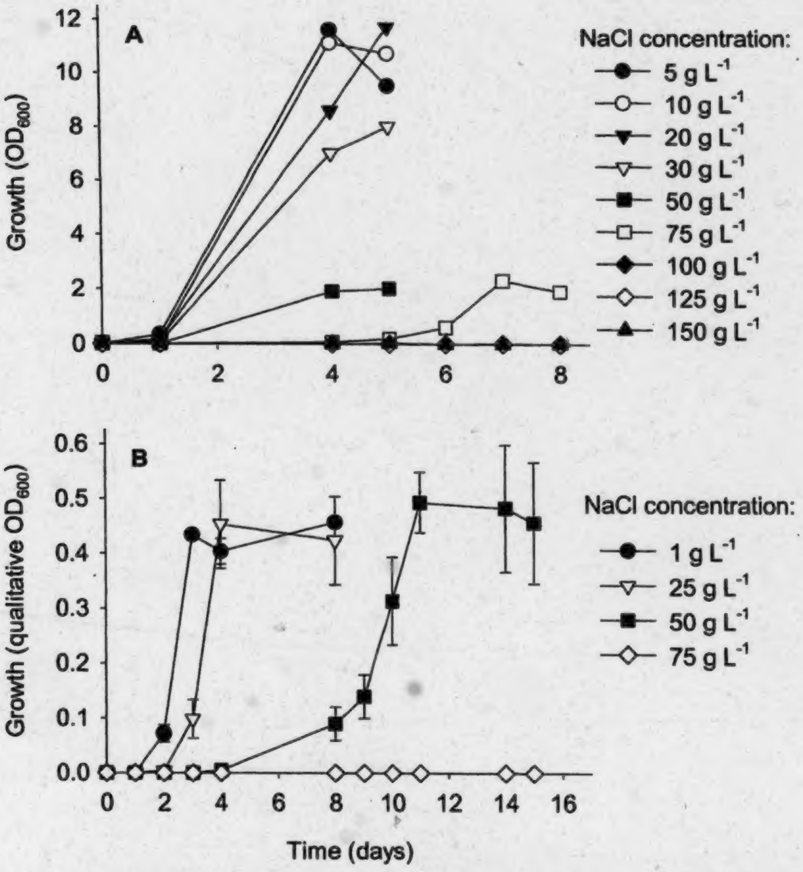

Figure 7.5. **Growth of *Rhodococcus* sp. strain JVH1 at different salt concentrations in: (A) TSB (n=1); and (B) SFMM+4Ac with 5 mmol sulfate L^{-1} (average ± standard deviation, n=3).**

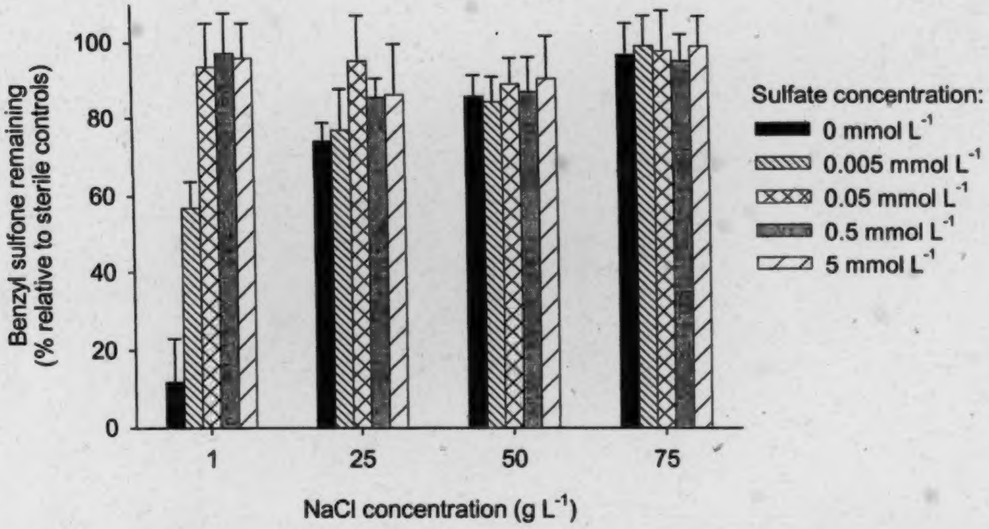

Figure 7.6. Effect of salt concentration on sulfate repression of benzyl sulfone degradation by *Rhodococcus* sp. strain JVH1 (average + standard deviation, n=3). Cultures were incubated for 8 days at the lower two salt concentrations, and for 15 days at the higher two salt concentrations.

7.3.2 Effect of sulfate analogues on sulfate repression

The sensitivity of *Rhodococcus* sp. strain JVH1 to the sulfate analogues selenate (SeO_4^{2-}), chromate (CrO_4^{2-}), molybdate (MoO_4^{2-}), and tungstate (WO_4^{2-}) was first tested in a rich medium (TSB), and then in a defined medium (SFMM+4Ac) with sulfate (1 mmol L^{-1}) as the sole sulfur source. JVH1 was able to grow in TSB with up to 1000 mmol L^{-1} of sulfate (positive control; data not shown), selenate, chromate, or molybdate (Figure 7.7A-C), and with up to 100 mmol L^{-1} of tungstate (Figure 7.7D). In the defined medium, JVH1 could grow with up to 100 mmol L^{-1} of added sulfate (positive control; data not shown), molybdate, or tungstate (Figure 7.8C,D). Selenate limited growth at 10 mmol L^{-1} (Figure 7.8A), and chromate prevented growth at 1 mmol L^{-1} (Figure 7.8B), suggesting that these compounds interfered with the ability of JVH1 to use sulfate.

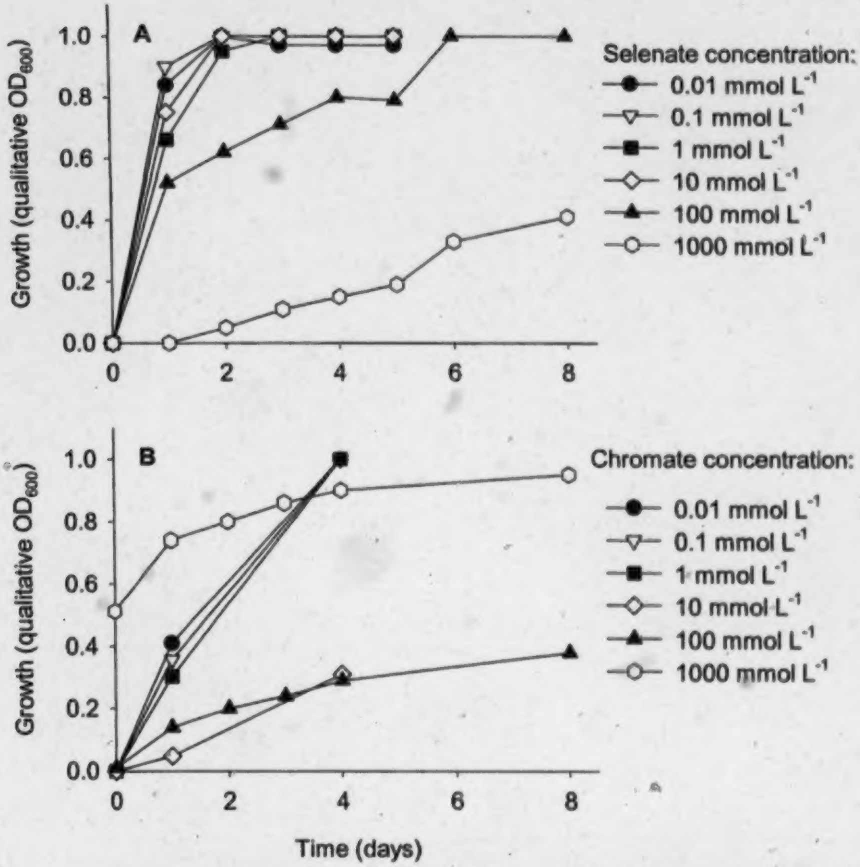

Figure 7.7. **Growth of *Rhodococcus* sp. strain JVH1 in TSB with different concentrations of sulfate analogues: (A) selenate; (B) chromate. In (B), the high optical densities observed at 1000 mmol chromate L^{-1} were due to the very dark colour of the chromate-TSB solution.**

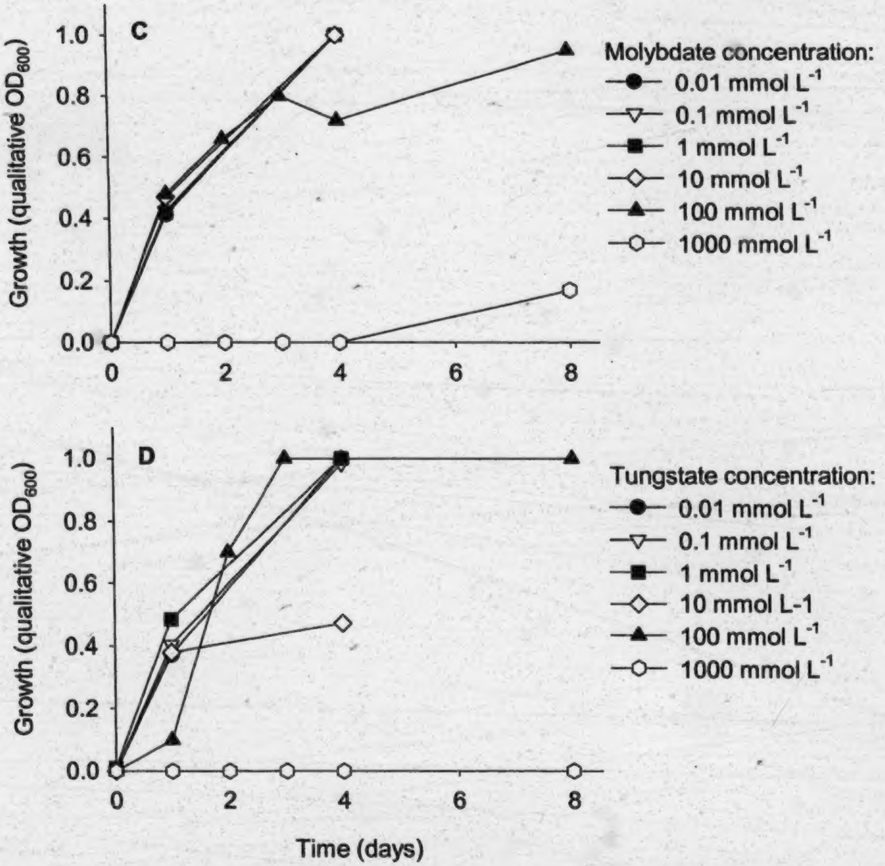

Figure 7.7 **(continued) Growth of *Rhodococcus* sp. strain JVH1 in TSB with different concentrations of sulfate analogues: (C) molybdate; (D) tungstate.**

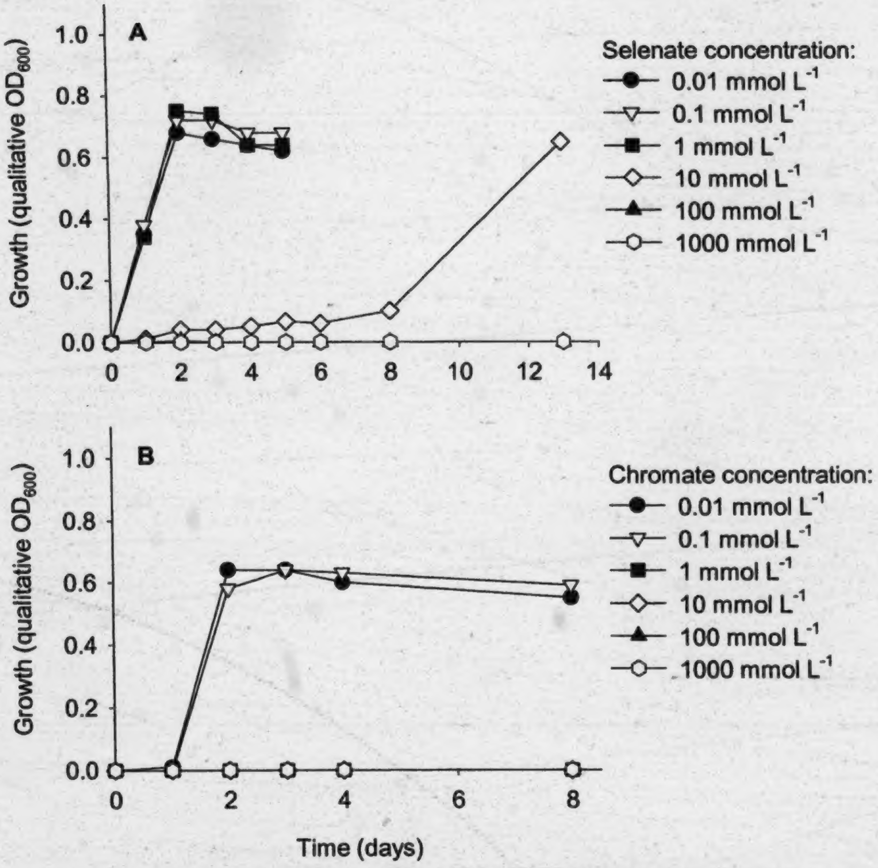

Figure 7.8. Growth of *Rhodococcus* sp. strain JVH1 in SFMM+4Ac with 1 mmol sulfate L^{-1} and different concentrations of sulfate analogues: (A) selenate; (B) chromate.

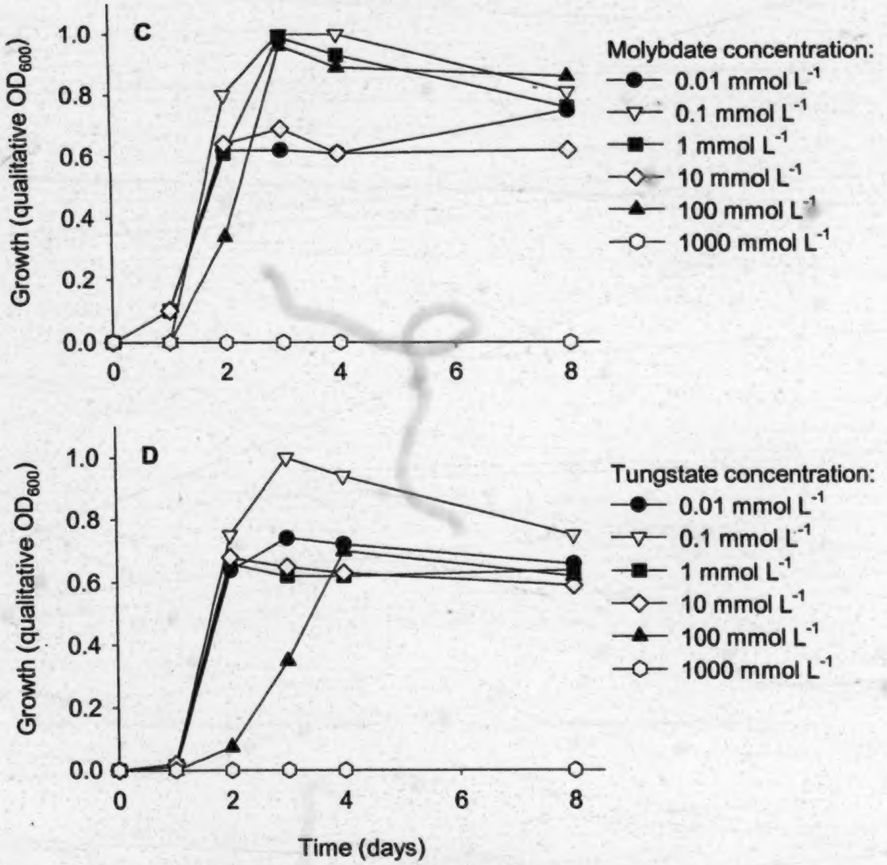

Figure 7.8 **(continued) Growth of *Rhodococcus* sp. strain JVH1 in SFMM+4Ac with 1 mmol sulfate L^{-1} and different concentrations of sulfate analogues: (C) molybdate; (D) tungstate.**

The effect of selenate and chromate on sulfate repression was tested by growing JVH1 with benzyl sulfone at different concentrations of sulfate with selenate or chromate. Cysteine was used instead of benzyl sulfone in some cultures as a positive control, because it is a product of sulfate metabolism that can be used for further cellular processes. Cysteine (50 μmol L^{-1}) allowed growth with 10 mmol L^{-1} of selenate (Figure 7.9A) or 1 mmol L^{-1} of chromate (Figure 7.10A). In addition, 5 mmol sulfate L^{-1} also allowed growth at 10 mmol selenate L^{-1} (Figure 7.9A) or 1 mmol chromate L^{-1} (Figure 7.10A).

Selenate had no effect on sulfate repression of the desulfurization of benzyl sulfone (Figure 7.9B). In an initial experiment with chromate, JVH1 grew with 1 mol chromate L^{-1} and 5 mmol sulfate L^{-1}, and around 50 % loss of benzyl sulfone was measured (Figure 7.10B). Two further experiments were performed to try and confirm this result. In the first, the effect of sulfate on loss of benzyl sulfone was measured without chromate and with 1 mmol chromate L^{-1}. The expected sulfate regulation profile was observed without chromate, but the addition of chromate did not allow degradation of benzyl sulfone in the presence of sulfate (Figure 7.11A). The second experiment tested the effect of incubation time. Loss of benzyl sulfone was measured with and without added sulfate and chromate after both 7 and 14 days. Chromate had no effect on sulfate regulation at either time point (Figure 7.11B). Because the results of the initial chromate experiment could not be repeated, it was concluded that chromate did not have an effect on sulfate regulation.

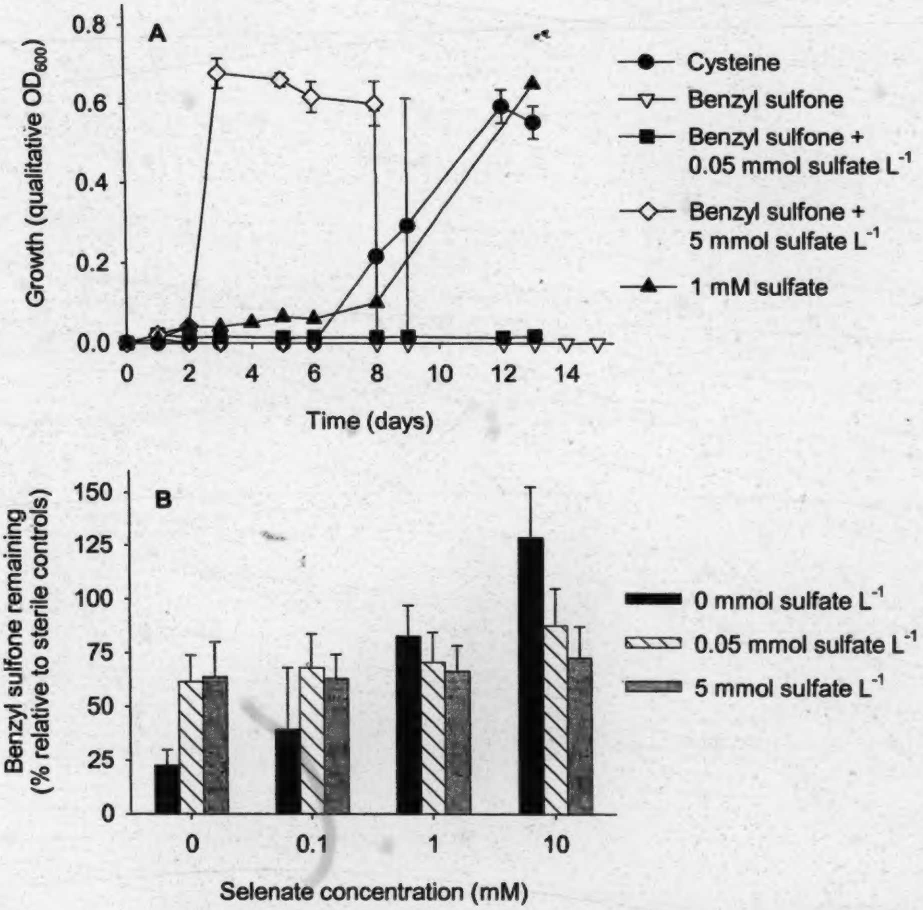

Figure 7.9. **(A) Growth of *Rhodococcus* sp. strain JVH1 with different sulfur sources at 10 mmol selenate L^{-1}. (B) Effect of selenate concentration on sulfate repression in *Rhodococcus* sp. strain JVH1. Cultures were incubated for 8 to 20 days, depending on growth (average ± standard deviation, n=3)**

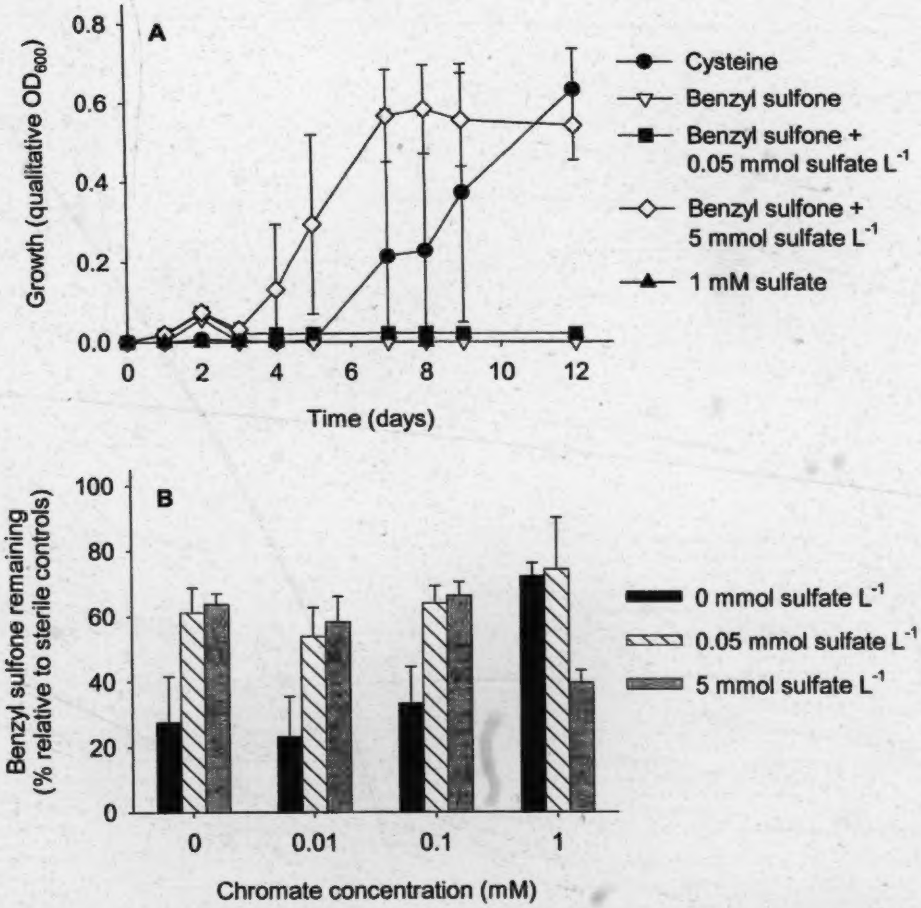

Figure 7.10. (A) Growth of *Rhodococcus* sp. strain JVH1 with different sulfur sources at 1 mmol chromate L^{-1}. (B) Effect of chromate concentration on sulfate repression in *Rhodococcus* sp. strain JVH1 after 12 days incubation. (average ± standard deviation, n=3)

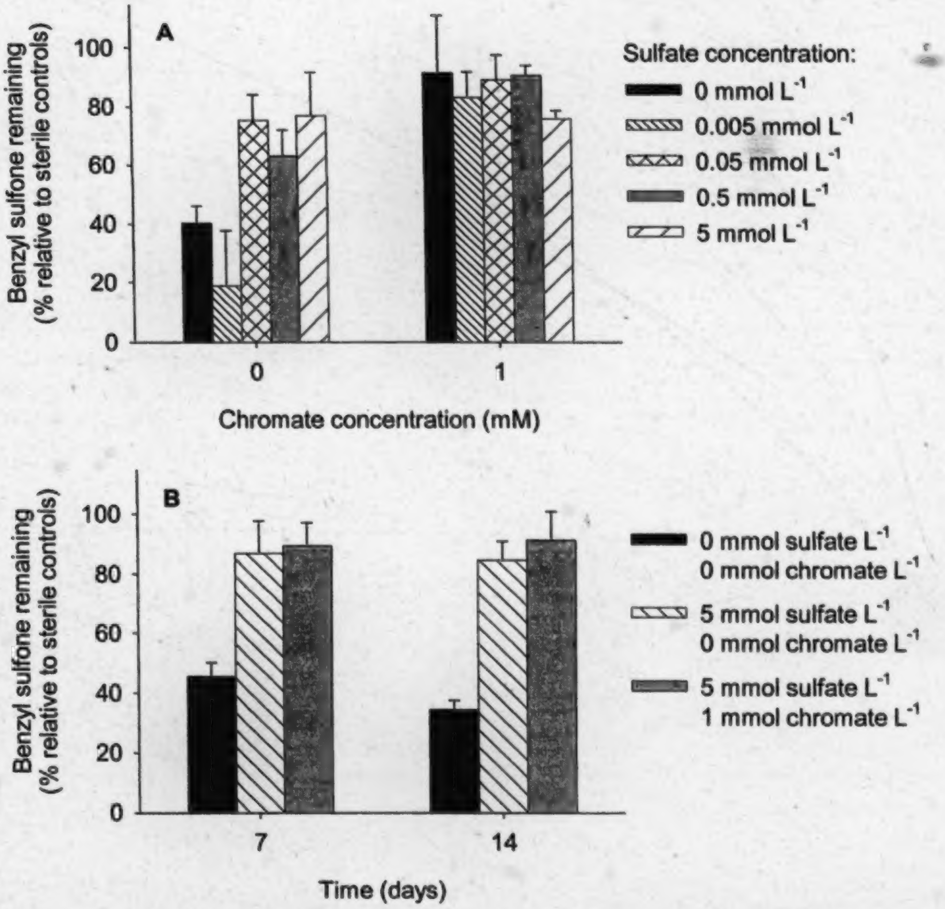

Figure 7.11. **Results of repeat experiments testing the effect of chromate on sulfate repression in *Rhodococcus* sp. strain JVH1: (A) first repeat experiment, showing the sulfate regulation profile at 0 mmol L^{-1} and 1 mmol L^{-1} of chromate after 6 days incubation; (B) second repeat experiment, showing the effect of incubation time on cultures with and without chromate. (average + standard deviation, n=3)**

7.4 Discussion and conclusions

The biotransformation of PFPS, 1,4-dithiane, benzyl sulfide, benzyl sulfoxide, benzyl sulfone, and *n*-octyl sulfide by *Rhodococcus* sp. strain JVH1 is subject to regulation by sulfate. This effect was seen directly on the disappearance of the parent compound for PFPS and 1,4-dithiane, but only on the accumulation or disappearance of the sulfone for benzyl sulfide, benzyl sulfoxide, benzyl sulfone, and *n*-octyl sulfide. This observation suggests that JVH1 is capable of both specific and non-specific oxidation of the sulfur atom, depending on the compound. As discussed in Section 4.9, sulfur oxidation may be catalysed by other oxidative enzymes, such as alkane monooxygenase [69]. The relative rates of specific and non-specific sulfoxidation in JVH1, and therefore the importance of non-specific oxidation to the desulfurization capabilities of the strain, cannot be assessed from the available data.

Loss of activity in the presence of sulfate is an undesirable trait, and further experiments were therefore undertaken to understand the regulatory mechanism and to explore means of alleviating this effect. Because transformation of aliphatic sulfides is not always affected by sulfate, an aliphatic sulfone is a more suitable model compound for these experiments (Figure 7.2). Benzyl sulfone was therefore used to study ways of alleviating sulfate regulation in JVH1.

Resting cell suspensions of *Rhodococcus* sp. strain JVH1 pre-grown with 1,4-dithiane, but not with sulfate, as the sole sulfur source were able to effect some disappearance of benzyl sulfone. This loss must be due to biological activity, rather than non-specific physical interactions with the cells, because no loss was observed in the killed controls. There was no difference in the loss of benzyl sulfone observed with or without sulfate added to the buffer, showing that sulfate does not inhibit the desulfurization enzymes. Sulfate therefore prevented expression of the desulfurization enzymes during growth of the cultures, consistent with a repression mechanism. This conclusion is consistent with the sulfate repression found in other bacteria metabolizing organosulfur compounds, such as dibenzothiophene [99], β-endosulfan [155], and taurine [159].

In some microorganisms, environmental conditions such as osmotic stress lead to increased sulfur requirements due to increased production of thiols such as glutathione

[38, 148]. *Rhodococcus* sp. strain JVH1 was tolerant of fairly high NaCl concentrations, growing with up to 75 g L^{-1} (1.3 mol L^{-1}) in a complex medium and with up to 50 g L^{-1} (0.9 mol L^{-1}) in a defined medium. These concentrations are well above what would typically be found in an oilfield brine, indicating that salinity would not limit JVH1 in a field upgrading operation. Comparable levels of halotolerance have been observed in other *Rhodococcus* isolates [88, 89, 105, 110, 127, 176], as well as in other hydrocarbon-degrading cultures [88, 104, 127, 176].

Rhodococcus sp. strain JVH1 was tested for sulfate repression at elevated salt concentrations. Although the cultures grew, no loss of benzyl sulfone was observed at the higher salt concentrations, regardless of the added sulfate concentration. In addition, the negative controls (no benzyl sulfone) at the higher salt concentrations grew as well as the cultures with an added sulfur source (not shown). It is likely therefore that sulfate contamination from the commercial NaCl supported growth and regulated benzyl sulfone desulfurization in these cultures. According to the supplier, the maximum sulfate content of the NaCl was 0.004 %, which would result in a sulfate concentration of 0.01 mmol L^{-1} in SFMM+4Ac containing 25 g NaCl L^{-1}. This concentration is sufficient to support growth of JVH1, and may be enough to significantly affect the use of organic sulfur sources. It has not been shown whether JVH1 can in fact cleave aliphatic sulfides at high salt concentrations. It is evident, however, that high salinity does not alleviate the regulatory effect of inorganic sulfate. There was, therefore, no need to repeat the experiment with a purer source of NaCl.

The sensitivity of *Rhodococcus* sp. strain JVH1 towards the sulfate analogues selenate, chromate, molybdate, and tungstate was tested to look for compounds interfering with sulfate utilization (transport or metabolism). Selenate and chromate both inhibited the growth of *Rhodococcus* sp. strain JVH1, whereas molybdate and tungstate had no effect. If the mechanism of selenate or chromate inhibition was due to interference with sulfate utilization, cysteine should allow growth by relieving the dependence of the cells on sulfate for sulfur. The addition of cysteine (50 μmol L^{-1}) restored growth of JVH1 with inhibitory concentrations of selenate (Figure 7.9A) and chromate (Figure 7.10A), showing that these compounds did in fact interfere with sulfate utilization by JVH1. In addition, increasing the sulfate concentration from 1 mmol L^{-1} to

5 mmol L^{-1} also allowed growth at 10 mmol selenate L^{-1} (Figure 7.9A) and 1 mmol chromate L^{-1} (Figure 7.10A), indicating that these two sulfate analogues interfered competitively with sulfate utilization. Chromate is taken up by bacterial sulfate transporters, and bacterial chromate resistance is often related to altered sulfate transport [146]. Competitive inhibition of sulfate uptake by chromate, and of chromate uptake by sulfate, has been shown in *Alcaligenes eutrophus* strain AE104 [117] and *P. fluorescens* strains LB300(pLHB1) and LB303 [119]. In addition, selenate and chromate resistance have been used to select sulfate transport mutants in the yeast *Saccharomyces cerevisiae* [25]. An interrelation between selenate and chromate toxicity and sulfur metabolism has not previously been reported for a *Rhodococcus* isolate.

Unlike cysteine, benzyl sulfone did not serve as an alternate sulfur source to support growth of *Rhodococcus* sp. strain JVH1 at elevated selenate and chromate concentrations. A minimum amount of sulfate, normally provided by low-level sulfate contamination of the mineral salts medium, may be required to initiate growth of JVH1 before the culture can start using benzyl sulfone. If sulfate transport is inhibited by selenate or chromate, that minimum sulfate concentration may not be available intracellularly. Alternately, adequate sulfate may enter the cells, but is not metabolized due to competitive inhibition by the sulfate analogues, preventing growth. In this case the intracellular sulfate concentration would be high, leading to repression of the desulfurization system for organic sulfur compounds. Finally, the cells may not be repressed and are able to metabolize benzyl sulfone, but cannot use the sulfite or sulfate released to grow due to the presence of the sulfate analogues. In the absence of growth, the overall conversion of benzyl sulfone would then be very low or undetectable. Chromate and selenate interfered with sulfate use, but their lack of effectiveness in alleviating the regulatory effect of sulfate on the desulfurization of benzyl sulfone by *Rhodococcus* sp. strain JVH1 could, therefore, be due to several possible mechanisms.

Experiments with high salt concentrations and with sulfate analogues were unsuccessful in alleviating sulfate repression in *Rhodococcus* sp. strain JVH1 under growth conditions. Another approach to controlling sulfate repression will therefore be required. One way to avoid repression is to grow cultures of JVH1 under sulfate-limited conditions to achieve high desulfurization activity, and use resting (non-growing) cells from these cultures for biocatalysis. The use of pre-grown resting cell suspensions may therefore be useful for circumventing the potentially negative effect of sulfate in field upgrading. Resting cells would need to be grown with an organic sulfur source under sulfate-limited conditions, however, preventing the use of inexpensive, rich media to generate high cell densities. Appropriate conditions, for example cofactor requirements, would also need to be determined to achieve high activity, allowing greater conversion of the substrate than was observed for benzyl sulfone. If sufficiently high activity cannot be achieved, genetic modification, such as promoter mutation or replacement, or introduction of desulfurization genes into an alternate host organism with appropriate regulatory sequences, may be necessary. This type of approach has been studied with the biodesulfurization of dibenzothiophene [45, 99, 142].

8. Overall discussion and conclusions

8.1 General discussion of the bacterial isolates studied

The work presented in this thesis was accomplished in two phases:

1. Establishing enrichment cultures selective for degradation of model compounds representing the aliphatic sulfide bridges found in asphaltenes, and characterizing bacterial isolates from the enrichment cultures to identify promising strains exhibiting sulfur-specific carbon-sulfur bond cleavage.
2. Characterizing the potential of the genetically unmodified microorganism *Rhodococcus* sp. strain JVH1 for biocatalytic upgrading to reduce crude oil viscosity.

In both phases of research, the same characteristics were of interest in the context of the biological upgrading application, and are discussed further here:

1. Substrate ranges of the isolates for both sulfidic and thiophenic organic sulfur compounds, and mechanisms of desulfurization.
2. Effect of sulfate on the desulfurization of organic sulfur compounds.
3. Activity of the isolates towards different types of hydrocarbons characteristic of crude oil.

8.1.1 Substrate range and desulfurization mechanism for organic sulfur sources

The substrate range of the isolates was of interest as an indicator of the potential activity towards the range of possible sulfur species in crude oil. The most desirable properties were a broad substrate range for aliphatic sulfur compounds, and no activity towards thiophenic sulfur compounds. This type of isolate would be expected to exhibit specific activity towards only the sulfide bridges in an oil.

The substrate range of selected isolates from enrichment cultures and of *Rhodococcus* sp. strain JVH1 was tested with a variety of aliphatic sulfur compounds. The compounds tested included dialkyl sulfides such as *n*-octyl sulfide, alicyclic sulfides such as 1,4-dithiane, and diaryl sulfides such as benzyl sulfide (Figure 4.1). The substrate range varied among the isolates. *R. erythropolis* strain EPWF and

Pseudomonas sp. strain K1oA were apparently only able to desulfurize 1,4-dithiane to support growth (Section 4.6), although EPWF was capable of sulfur oxidation in both *n*-octyl sulfide and benzyl sulfide (Sections 4.4, 4.7). *Rhodococcus* sp. strain K1bD grew with *n*-octyl sulfide, 1,4-dithiane, and benzyl sulfide as sulfur sources (Sections 4.4, 4.6, 4.7). *Rhodococcus* sp. strain JVH1 was tested with a wider selection of compounds, and was able to use several types of dialkyl, alicyclic, and diaryl sulfides as sulfur sources (Table 5.1).

Of the 22 isolates from the sulfur-limited enrichment cultures that were active towards at least one aliphatic sulfide, nine were also able to use the thiophenic compound dibenzothiophene as a sulfur source, and the other 13 were not (Table 4.3). *Rhodococcus* sp. strain JVH1 did not use dibenzothiophene, but could use benzothiophene and a variety of alkylbenzothiophenes as sulfur sources (Section 5.3). These results do not confirm or deny an interrelation between the desulfurization of sulfidic and thiophenic compounds, but do show that the ability to desulfurize one class of compound does not necessarily indicate activity towards the other class, or even towards other compounds from the same class.

Identification of metabolites from the desulfurization of organic sulfur compounds allowed a functional comparison between the enzyme systems of *Rhodococcus* sp. strain K1bD and *Rhodococcus* sp. strain JVH1. The same types of metabolites were produced from different compounds by both K1bD (Sections 4.4, 4.7, 4.8) and JVH1 (Table 5.1, Table 5.4). The metabolites identified included sulfones from benzyl sulfide, *n*-octyl sulfide, and benzothiophenes (JVH1 only); sulfinates (identified as sultines) from benzothiophenes (JVH1 only); alcohols from benzyl sulfide (K1bD only), dibenzothiophene (K1bD only), and benzothiophene (JVH1 only); and benzoic acid from benzyl sulfide. These metabolites were consistent with the pathway for PFPS desulfurization in *Rhodococcus* sp. strain JVH1 (Figure 2.6) [160], the 4S pathway for dibenzothiophene desulfurization in *R. erythropolis* strain IGTS8 (Figure 2.3B) [49] and other organisms, and to the known pathways for desulfurization of benzothiophenes in different organisms (Figure 2.4) [47, 92]. This pattern showed that functionally similar enzyme systems were used by K1bD and JVH1 for desulfurization of various substrates, and suggested that these systems were also at least functionally related to known systems for the desulfurization of organic sulfur compounds in other organisms. The desulfurization enzymes may also be genetically related, but this conclusion cannot be directly drawn from the available data.

8.1.2 Response to inorganic sulfate

Inorganic sulfate had a negative effect on the desulfurization of organic sulfur compounds by all four isolates tested (*R. erythropolis* strain EPWF, *Rhodococcus* sp. strain K1bD, *Pseudomonas* sp. strain K1oA, and *Rhodococcus* sp. strain JVH1) (Figure 4.7, Figure 5.5, Figure 7.1, Figure 7.2, Figure 7.3). This regulatory effect was shown to be repression in strain JVH1. Sulfate repression needs to be alleviated, because sulfate could be present in the water used for field upgrading, and because sulfate would be produced through desulfurization of the oil and could therefore limit the extent of conversion.

Sulfur oxidation was not always affected by sulfate. For example, the oxidation of benzyl sulfide to benzyl sulfone occurred at high sulfate concentrations in cultures of *R. erythropolis* strain EPWF, *Rhodococcus* sp. strain K1bD, and *Rhodococcus* sp. strain JVH1 (Figure 4.8, Figure 7.2A). Sulfur oxidation to the sulfone was likely catalysed non-specifically by unrelated oxidative enzymes in these organisms. Carbon-sulfur bond cleavage, however, appears to be consistently regulated by sulfate. The conversion of benzothiophene sulfone and of benzyl sulfone by JVH1 was repressed at high sulfate concentrations (Figure 5.5, Figure 7.2C). The carbon-sulfur bond cleavage reactions must therefore depend solely on specific enzyme-catalysed reactions.

Resting-cell cultures of *Rhodococcus* sp. strain JVH1 grown with an organic sulfur source were active in the presence of sulfate (Figure 7.4), giving one means of circumventing sulfate repression. The extent of conversion was not as high as in growing cultures, however, and the requirement of defined media for growth of the cultures could impose prohibitive expenses in an industrial process. Selenate and chromate were shown to interfere with sulfate use, but did not allow the use of benzyl sulfone as an alternate sulfur source (Figure 7.9, Figure 7.11). Another means of removing sulfate repression, for example through genetic modification, would be required for further development using this culture.

8.1.3 Activity towards hydrocarbons

n-Alkane degradation was observed in all but one of the active isolates from the enrichment cultures (Section 4.3.1, Table 4.3), as well as in *Rhodococcus* sp. strain JVH1 (Section 5.4.1). JVH1 was also able to degrade the isoprenoid alkane pristane and monoterminally-substituted alkanes (such as 1-phenyldodecane) as carbon sources, and was capable of cometabolic degradation of the highly branched alkane HMN, which did not serve as a carbon source. This property is problematic for upgrading, because loss of hydrocarbons reduces the fuel value of the oil. JVH1 also degraded the *n*-alkanes in Lloydminster crude oil (Figure 6.5). Alkane degradation was not subject to catabolite repression in JVH1 (Figure 5.8), so another mechanism will be required to control this undesirable trait in the development of an upgrading process.

8.2 Overall conclusions and suggestions

Based on the two phases of this work, overall conclusions can be drawn with respect to the effectiveness of the enrichment technique using commercially available aliphatic sulfides as model compounds to represent the sulfide linkages in crude oil, and on the suitability of *Rhodococcus* sp. strain JVH1 for the biological upgrading of crude oil to achieve viscosity reduction through cleavage of aliphatic sulfide bridges in the oil. The bacteria studied in this research may also have useful biocatalytic properties for applications outside of crude oil upgrading.

8.2.1 Effectiveness of enrichment cultures using commercially available aliphatic sulfides as model compounds

The model compounds *n*-octyl sulfide, *n*-octadecyl sulfide, and 1,4-dithiane were selected to represent the aliphatic sulfide linkages in asphaltenes, and were used to establish sulfur-limited and carbon-limited enrichment cultures. The dialkyl sulfides could serve as either a sulfur source or as a carbon and sulfur source, whereas 1,4-dithiane only served as a sulfur source. The enrichment cultures yielded a number of bacteria active towards aliphatic sulfur compounds, but were not very effective for selecting bacteria with the desired sulfur-specific activity. Two main challenges were identified for this type of enrichment strategy: the relatively low sulfur requirements for bacterial growth, and the presence of multiple pathways for the degradation of the model compounds.

Aerobic, heterotrophic bacteria have relatively low sulfur requirements for growth, compared to carbon requirements. Growth, assessed as an increase in turbidity, was observed to some degree in all the sulfur-limited enrichment cultures, and was maintained through successive transfers. Careful screening procedures were needed to identify which cultures (both mixed and pure cultures) were in fact dependent on the model compounds for their sulfur requirements. In addition, the enrichment cultures maintained a large population of strains that were not individually active towards the model compounds, indicating that the few active strains were able to release enough sulfur to maintain a large mixed population. Problems were also encountered with low-level impurities in the commercial preparations of the model compounds supporting significant growth of isolated bacterial strains, necessitating further purification of the compounds.

The model compounds were not selective for only the desired, sulfur-specific attack, but instead were subject to more than one mechanism of degradation. As a result, for some cultures growth and loss of substrates were observed, but were not in fact indicative of desirable biotransformations. *n*-Octyl sulfide, in particular, was subject to terminal degradation analogous to the biodegradation of *n*-alkanes, as well as sulfur oxidation. Non-specific sulfur oxidation of sulfides to sulfones also gave loss results that were not indicative of carbon-sulfur bond cleavage and sulfur release.

A small proportion of the isolated bacteria did grow with at least one of the model compounds serving as the sole sulfur source. To maximize the effectiveness of sulfur-limited enrichment cultures, model compounds should be selected to provide as few avenues of attack as possible. For the case of aliphatic sulfide bridges, the use of compounds such as PFPS [160] would be preferable to the commercially available options: because the only opportunity for attack is at the sulfur bridge, greater selective pressure is exerted on the enrichment cultures, and screening is more direct. In any case, careful screening against sulfur-free negative controls, and early identification of metabolites, are essential to rapid and accurate identification of interesting strains.

8.2.2 Suitability of *Rhodococcus* sp. strain JVH1 for biological upgrading of crude oil

Rhodococcus sp. strain JVH1 was selected as a promising candidate for the biological upgrading application because it demonstrated a sulfur-specific pathway for the use of the aliphatic sulfide PFPS, it used a broad range of aliphatic sulfur compounds as sulfur sources for growth, and it did not use thiophenic sulfur compounds [160]. Further work with JVH1 in this project identified some potential concerns with the organism: JVH1 did use benzothiophene and several alkylbenzothiophenes as sulfur sources, JVH1 degraded alkanes as carbon sources, and the desulfurization of organic sulfur compounds was subject to sulfate repression.

The removal of sulfur from thiophenic structures such as benzothiophenes is desirable for general desulfurization during oil upgrading, which was not the primary goal here. Instead, a selective attack on aliphatic sulfur bridges was desired, as these structures are the only sulfur groups whose cleavage could lead to a reduction in the molecular size of asphaltenes. Competitive inhibition was observed between benzothiophene and benzyl sulfide, and between benzothiophene sulfone and benzyl sulfone, showing that the same enzyme system was used for both aliphatic and thiophenic compounds. Some activity towards two-ring thiophenic structures will therefore be unavoidable with the desulfurization system from *Rhodococcus* sp. strain JVH1.

Alkane degradation is not acceptable because hydrocarbons supply the fuel value of an oil. *Rhodococcus* sp. strain JVH1 degraded *n*-alkanes of different chain lengths from *n*-octane on vapour plates up to *n*-pentacosane in crude oil. An increase in the viscosity of the crude oil was also attributed to the removal of the *n*-alkanes by JVH1. The degradation of *n*-hexadecane was not subject to control through catabolite repression.

Inorganic sulfate caused repression of the desulfurization system in *Rhodococcus* sp. strain JVH1, which would limit the activity of JVH1 in field upgrading. The sulfate analogues selenate and chromate were effective in preventing sulfate use by the culture, inhibiting growth, but although cysteine could be used to restore growth, benzyl sulfone was not used. Resting cell suspensions were active towards benzyl sulfone, but the extent of degradation was low compared to growing cultures.

Genetic modifications may be the best way to optimize the activity of *Rhodococcus* sp. strain JVH1 and eliminate alkane degradation and sulfate repression.

Two different approaches could be effective. First would be to directly modify the JVH1 strain. Promoter mutation or replacement would be required to remove sulfate repression. All genes for alkane degradation would also have to be knocked out. Alternately, the desulfurization genes could be isolated and introduced into a different microorganism, with an appropriate promoter to avoid sulfate repression. In this case, the microorganism would be selected with no known hydrocarbon degradation capabilities. The importance of other characteristics of JVH1 to its desulfurization ability would need to be assessed, for example the hydrophobicity of the microorganism, which allows adhesion to the organic phase. Although adhesion is not required for the use of small compounds with some aqueous solubility, such as benzothiophene and benzyl sulfide, it may be required for the use of larger compounds (like asphaltenes) that are essentially only present in the oil phase. Despite these limitations, the specific desulfurization pathway of *Rhodococcus* sp. strain JVH1 for desulfurization of aliphatic sulfur groups still has potential for the development of a biological upgrading process and is still therefore of interest.

8.2.3 Other applications for the aliphatic-sulfide-degradading isolates studied

Rhodococci are recognized for their metabolic diversity, with members of this genus able to biotransform a wide range of organic compounds [30, 167]. The rhodococcal strains studied in this research were able to non-specifically oxidize the sulfur in aliphatic sulfides, specifically oxidize sulfur and cleave carbon-sulfur bonds in sulfidic and thiophenic compounds, and degrade aliphatic hydrocarbons. These abilities, in particular those not regulated by inorganic sulfate, may find application in more traditional biodegradation strategies, such as the disposal of sulfur mustard or the bioremediation of organic sulfur compounds from petroleum.

Sulfoxidation is also an important reaction in organic synthesis. Oxidation of asymmetric sulfides yields chiral sulfoxides, which can confer chirality to neighbouring carbon atoms in subsequent reaction steps [56]. Enzymatic sulfoxidation has been shown to offer high yields with varying optical purity depending on the enzymes and substrates used (reviewed in [55-57]). Among the bacterial oxygenases studied for sulfoxidation are alkane monooxygenase from *Pseudomonas oleovorans* [69], cyclohexanone monooxygenase from *Acinetobacter* sp. strain NCIB 9871 [24], and dibenzothiophene monooxygenase (DszC) from *R. erythropolis* strain BKO-53 (a derivative of strain IGTS8 [4]) [58, 59]. Both the specific and non-specific sulfoxidation abilities of *Rhodococcus* sp. strain JVH1 and the other isolates studied could therefore be of practical value for research in enzymatic organic synthesis.

9. References

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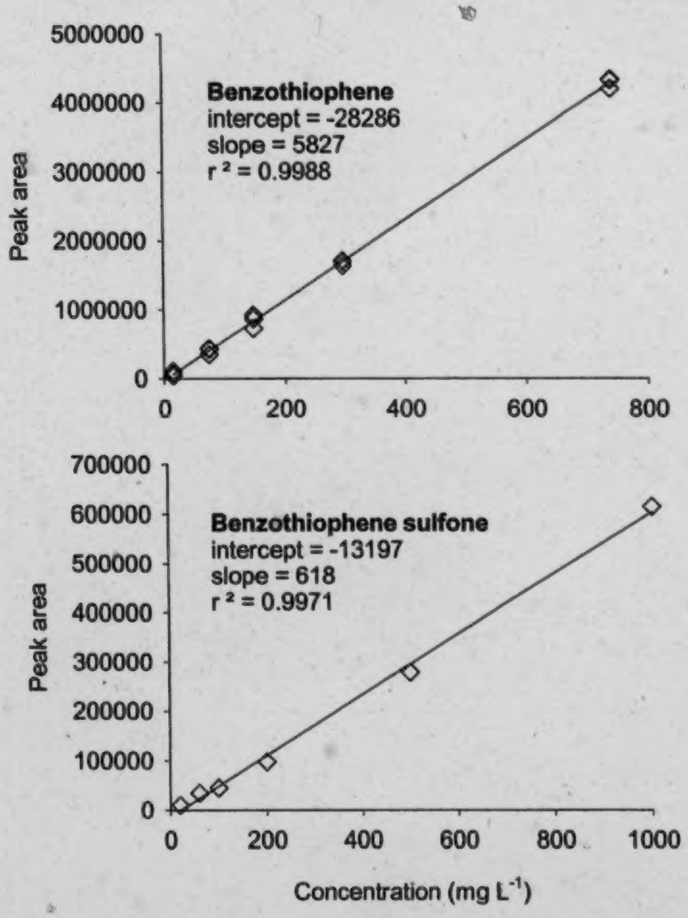
Appendix A Standard curves and calculations for GC-FID analyses

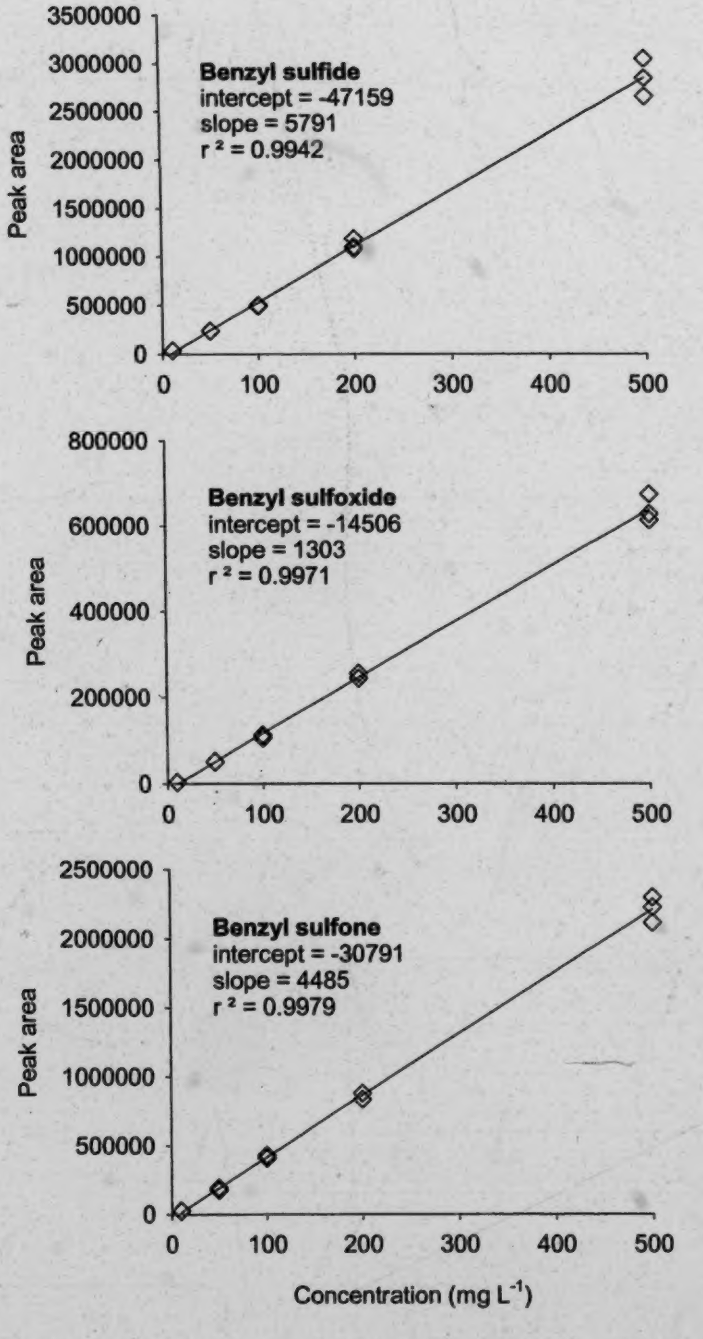
This appendix presents sample standard curves generated for GC-FID quantitation of organic compounds, showing the linear relationship between the mass concentration of the sample and the resulting peak area. Calculation procedures are also given for quantitation of compounds with and without standard curves.

A.1 Standard curves for organic sulfur compounds

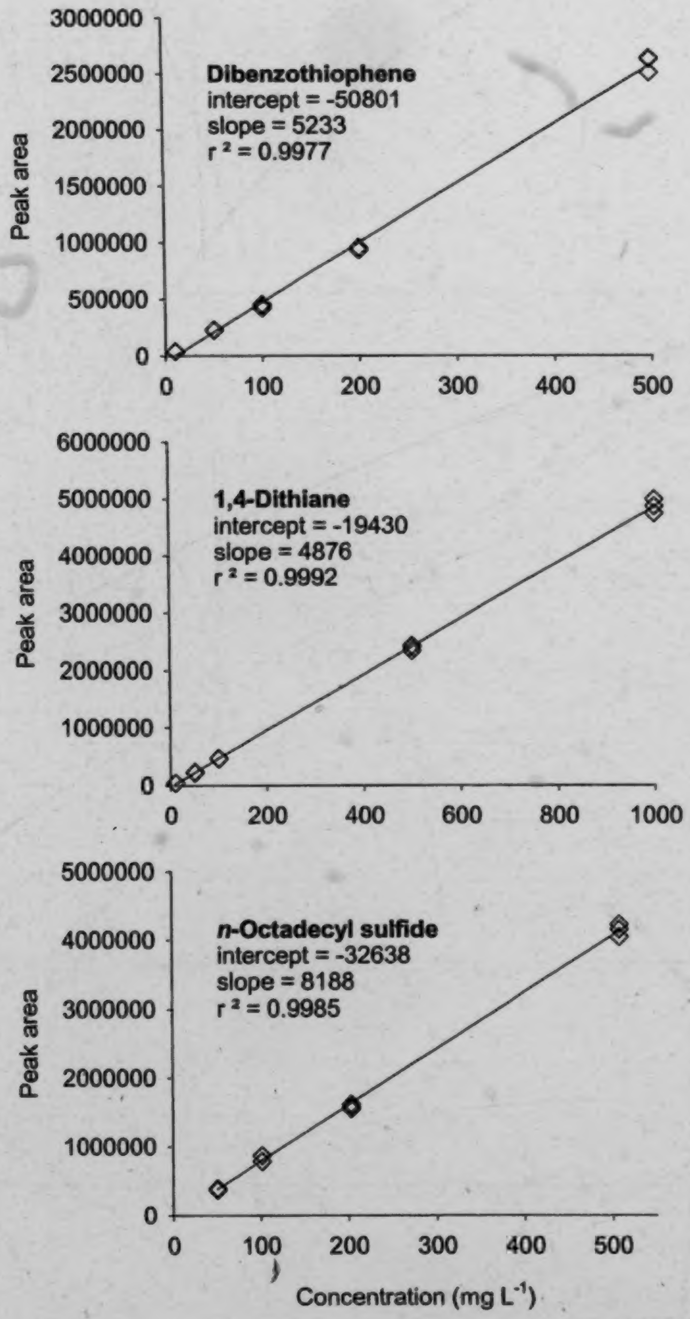
The standard curves in this section were obtained by using a Hewlett-Packard 5890 series GC, equipped with a 25-m HP-1 capillary column (Agilent Technologies).



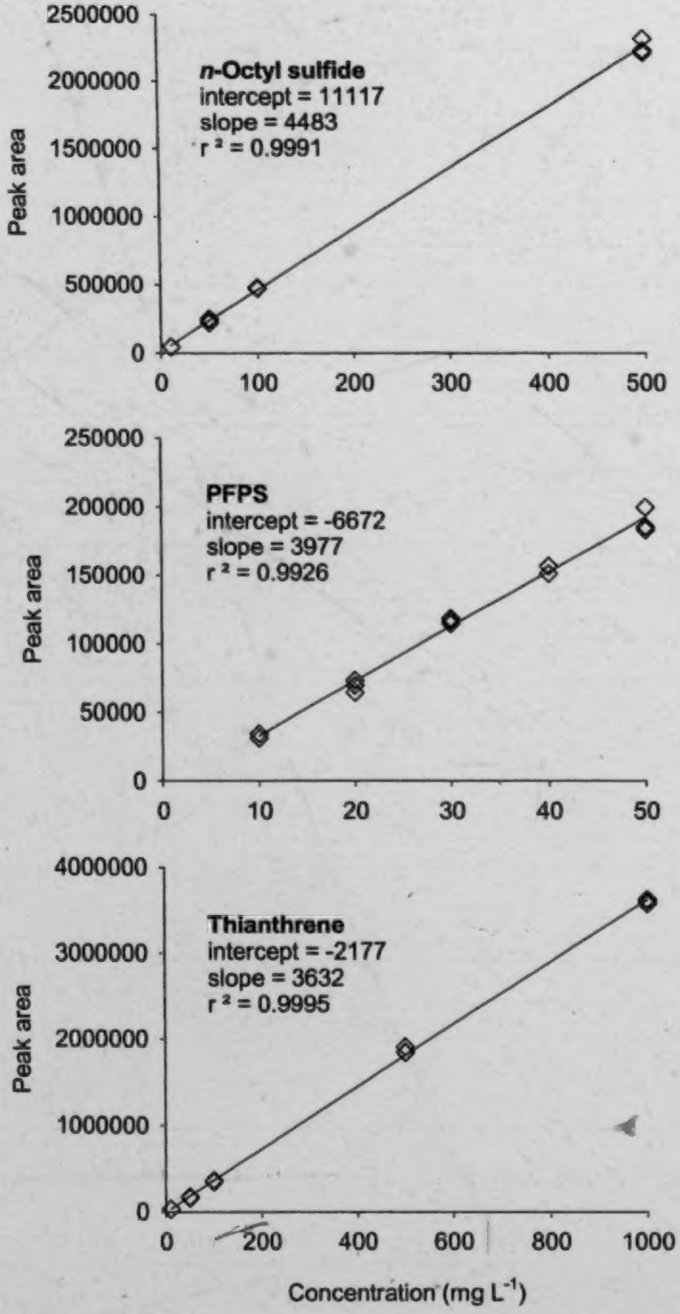
Sample standard curves for benzothiophene and benzothiophene sulfone.



Sample standard curves for benzyl sulfide, benzyl sulfoxide, and benzyl sulfone.



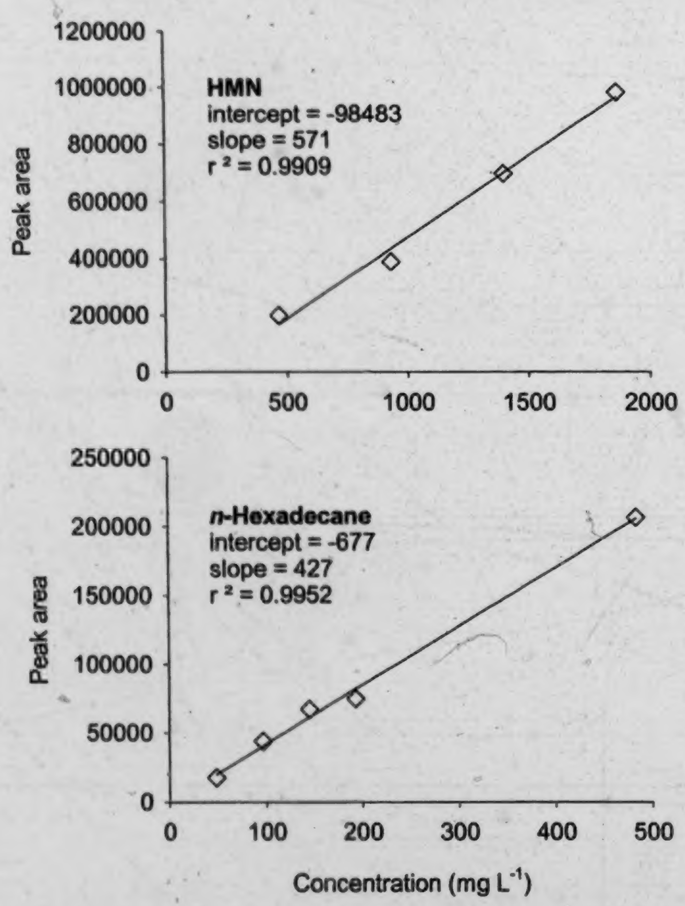
Sample standard curves for dibenzothiophene, 1,4-dithiane, and *n*-octadecyl sulfide.



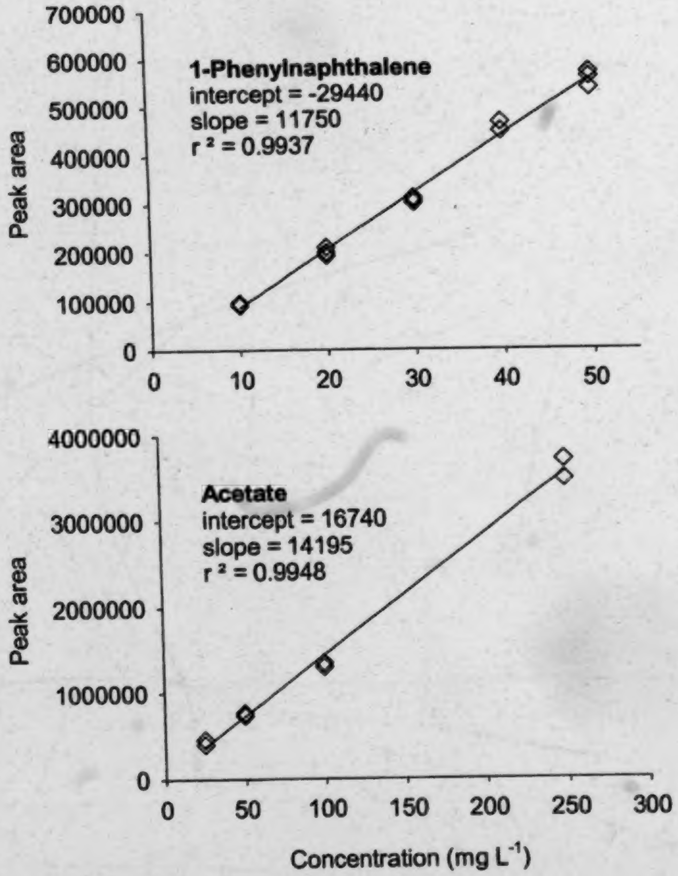
Sample standard curves for *n*-octyl sulfide, PFPS, and thianthrene.

A.2 Standard curves for hydrocarbons and acetate

The standard curves in this section were obtained using a Hewlett-Packard 5890 series GC, equipped with a 25-m HP-1 capillary column (Agilent Technologies) (hydrocarbons), or a 30-m DB-FFAP capillary column (Agilent Technologies) (acetate).



Sample standard curves for HMN and *n*-hexadecane.



Sample standard curves for 1-phenylnaphthalene and acetate.

A.3 Calculations for extracted compounds with standard curves

1. Calculation of concentrations in the cultures and sterile controls

For a compound, i, with peak area A_i:

$$C_{i,extract} = \frac{A_i - b}{m}$$

where: $C_{i,extract}$ is the concentration of compound i in the extract, mg L^{-1}
A_i is the peak area of compound i from the GC-FID chromatogram
b is the intercept of the standard curve for compound i
m is the slope of the standard curve for compound i

Using an internal standard, the concentration of a compound in the culture, $C_{cpd,culture}$, in mg L^{-1}, is:

$$C_{cpd,culture} = \frac{C_{istd,culture}}{C_{istd,extract}} \times C_{cpd,extract}$$

where: $C_{istd,culture}$ is the known concentration of internal standard in the culture, mg L^{-1}
$C_{istd,extract}$ is the calculated concentration of internal standard in the extract, mg L^{-1}
$C_{cpd,extract}$ is the calculated concentration of the compound in the extract, mg L^{-1}

The average concentration among a set of replicate cultures, $C_{avg,culture}$, in mmol L^{-1}, is:

$$C_{avg,culture} = \frac{\sum_{j=1}^{n} C_{cpd,culture,j}}{n \times MW_{cpd}}$$

where n is the number of replicates
MW_{cpd} is the molecular weight of the compound, mg $mmol^{-1}$

Note: The same calculations were used to find the concentration of compounds in the sterile controls ($C_{cpd,control}$ and $C_{avg,control}$).

2. Calculation of relative amounts remaining in or lost from the cultures

$$\% \text{ Left} = \frac{C_{avg,culture}}{C_{avg,control}} \times 100\%$$

$$\% \text{ Lost} = 100\% - \% \text{ Left}$$

$$SD = \sqrt{SD_{culture}^{\;2} + SD_{control}^{\;2}}$$

where: SD is the error, propagated from the standard deviations of the concentrations, and represents the overall standard deviation

$SD_{culture}$ is the standard deviation associated with $C_{avg,culture}$

$SD_{control}$ is the standard deviation associated with $C_{avg,control}$

A.4 Calculations for extracted compounds without standard curves

1. Calculation of peak area ratios for the cultures and sterile controls

Standard curves were not generated for all the compounds tested in the substrate range experiment, or for the alkylbenzothiophenes tested. Instead, peak area ratios were used to estimate the amount of a compound remaining in the cultures. Assuming that the peak area is directly proportional to the mass concentration of a sample and that the extraction efficiency was the same for the compound of interest and the internal standard:

$$A_{R,culture} = \frac{A_{cpd}}{A_{istd}} \approx \frac{C_{cpd,extract}}{C_{istd,extract}} \approx \frac{C_{cpd,culture}}{C_{istd,culture}}$$

where: $A_{R,culture}$ is the peak area ratio of the compound to the internal standard

A_{cpd} is the peak area of the compound from the GC-FID chromatogram

A_{istd} is the peak area of the internal standard from the GC-FID chromatogram

$C_{cpd,extract}$ is the mass or molar concentration of the compound in the extract

$C_{istd,extract}$ is the mass or molar concentration of the internal standard in the extract

$C_{cpd,culture}$ is the mass or molar concentration of the compound in the culture

$C_{istd,culture}$ is the mass or molar concentration of the internal standard in the culture

The average peak area ratio, $A_{R,avg,culture}$, is:

$$A_{R,avg,culture} = \frac{\sum_{j=1}^{n} A_{R,culture,j}}{n}$$

where: n is the number of replicates

Note: The same calculations were used to find the peak area ratios for the sterile controls ($A_{R,control}$ and $A_{R,avg,control}$).

2. Calculation of relative amounts remaining in or lost from the cultures

The concentration of the internal standard was the same in the cultures and in the sterile controls.
For compounds where replicate sterile controls were used:

$$\%\ \text{Left} = \frac{A_{R,avg,culture}}{A_{R,avg,control}} \times 100\%$$

$$\%\ \text{Lost} = 100\% - \%\ \text{Left}$$

$$SD = \sqrt{SD_{culture}^{2} + SD_{control}^{2}}$$

where: SD is the error, propagated from the standard deviations of the peak area ratios, and represents the overall standard deviation
$SD_{culture}$ is the standard deviation associated with $A_{R,avg,culture}$
$SD_{control}$ is the standard deviation associated with $A_{R,avg,control}$

For compounds where a single sterile control was used:

$$\%\ \text{Left} = \frac{A_{R,avg,culture}}{A_{R,control}} \times 100\%$$

$$\%\ \text{Lost} = 100\% - \%\ \text{Left}$$

$$SD = SD_{culture}$$

A.5 References

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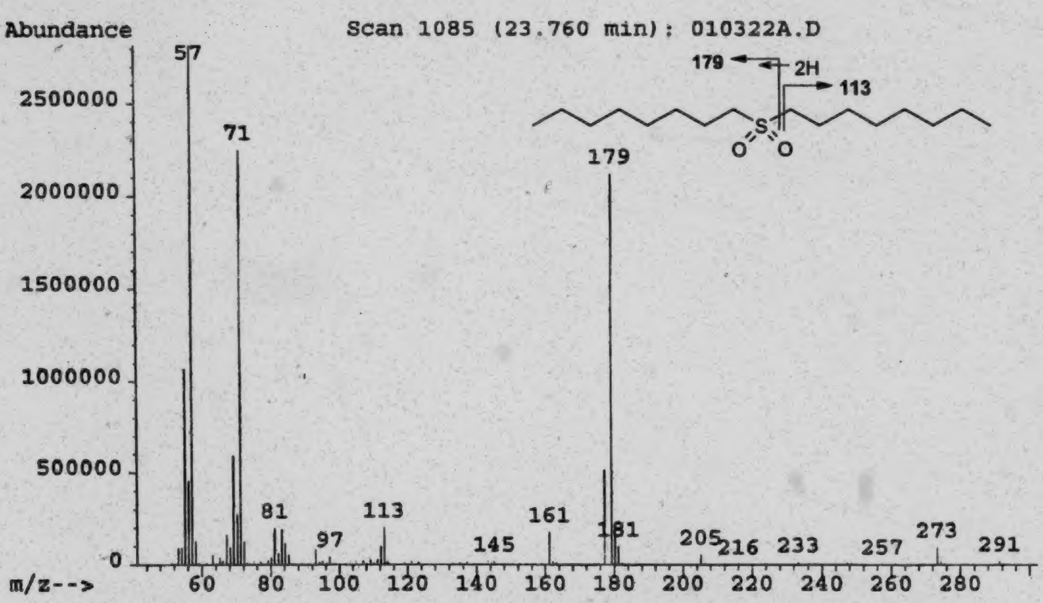
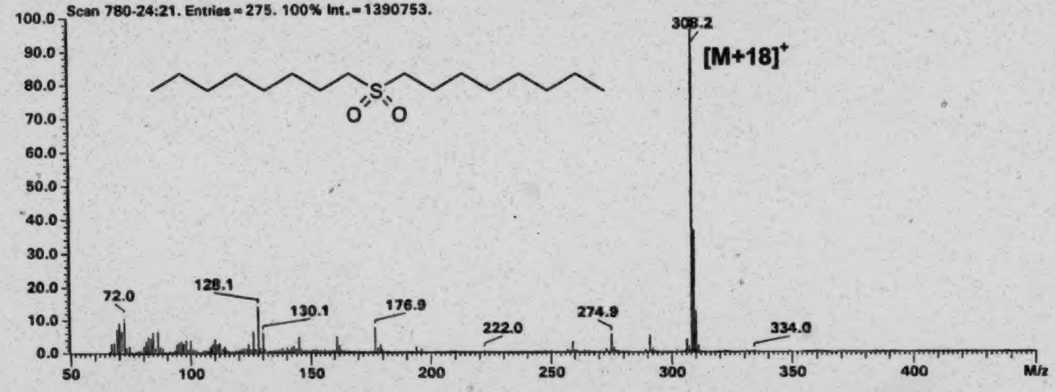
Appendix B Mass and FTIR spectra of metabolites of *n*-octyl sulfide

This appendix presents the mass and FTIR spectra used for identification of the metabolites of *n*-octyl sulfide produced by *R. erythropolis* strain EPWF, *Rhodococcus* sp. strain K7b1, *Rhodococcus* sp. strain K1bD, and *Pseudomonas* sp. strain K1oA.

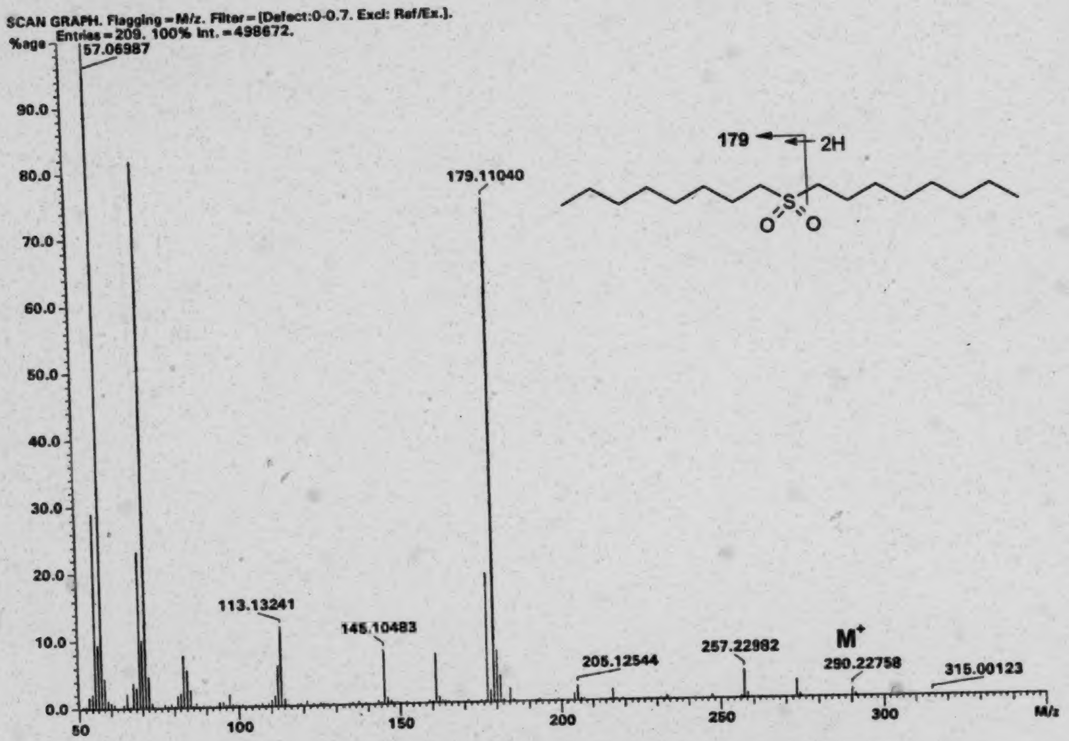
B.1 Octyl sulfone



Low resolution electron impact mass spectrum for octyl sulfone [GC-MS (EI)].



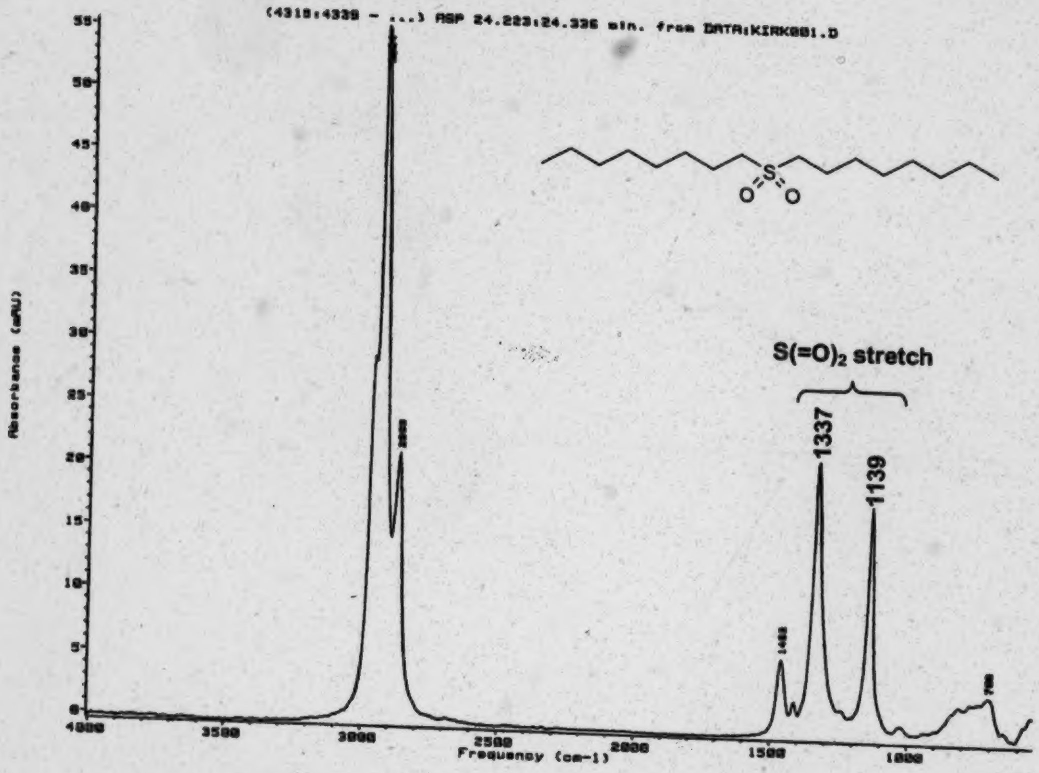
Chemical ionization mass spectrum for octyl sulfone [GC-MS (NH_4^+ CI)].



Mass	%age	Calculated	ppm	mmu	R/DB	Formula
290.22758	1.24	290.22797	1.3	0.4	0.0	$C_{16}H_{34}O_2S$
273.22488	2.56	273.22522	2.0	0.6	0.5	$C_{16}H_{33}OS$
258.23246	0.77	— No match found —				
257.22982	4.16	257.23025	1.9	0.5	0.5	$C_{16}H_{33}S$
233.15698	0.38	233.15753	2.4	0.6	0.5	$C_{12}H_{25}O_2S$
216.00614	1.74	216.00674	2.8	0.6	9.0	$C_{12}H_8S_2$
205.12544	2.26	205.12622	3.8	0.8	0.5	$C_{10}H_{21}O_2S$
204.11712	1.15	— No match found —				
184.03457	1.86	184.03467	0.5	0.1	9.0	$C_{12}H_8S$
181.10733	3.89	— No match found —				
180.11340	7.85	— No match found —				
179.11040	75.24	179.11058	1.0	0.2	-0.5	$C_9H_{19}O_2S$
179.09044	1.05	— No match found —				
178.09900	1.77	— No match found —				
177.09466	19.13	177.09492	1.6	0.3	0.5	$C_9H_{17}O_2S$
162.10433	0.85	162.10446	0.8	0.1	5.0	$C_{11}H_{14}O$
161.09957	7.35	161.10001	2.7	0.4	0.5	$C_9H_{17}OS$
147.11722	0.51	147.11737	1.0	0.2	4.5	$C_{11}H_{15}$
146.10836	0.89	— No match found —				
145.10483	7.31	145.10510	1.9	0.3	0.5	$C_9H_{17}S$
121.03151	0.63	— No match found —				
114.13583	1.08	— No match found —				
113.13241	11.81	— No match found —				
112.12488	9.02	112.12520	2.8	0.3	1.0	C_8H_{16}

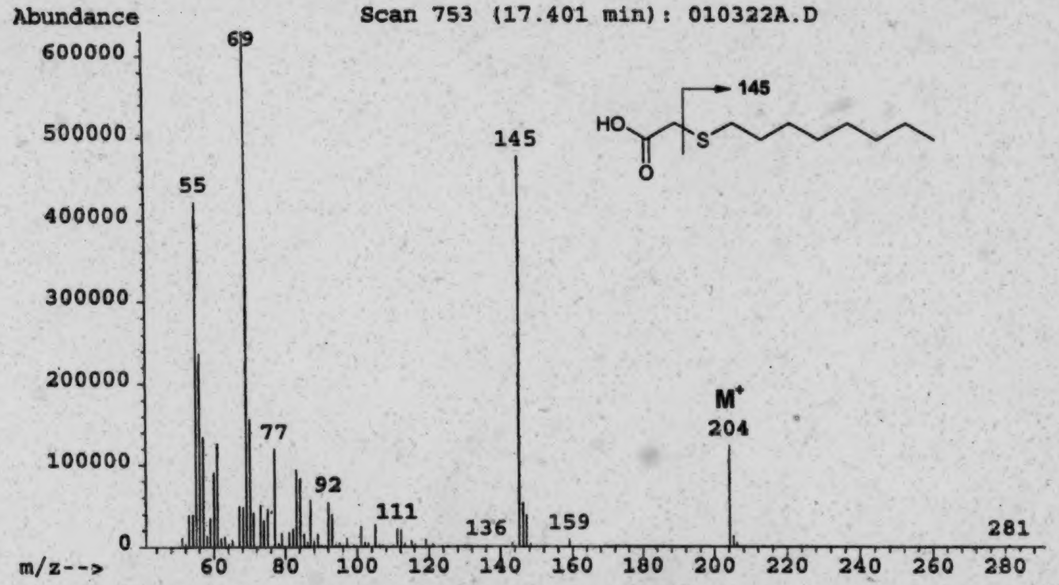
Mass	%age	Calculated	ppm	mmu	R/DB	Formula
111.11701	1.55	111.11738	3.3	0.4	1.5	C_8H_{15}
110.10922	0.96	110.10955	3.0	0.3	2.0	C_8H_{14}
109.10162	0.51	109.10172	0.9	0.1	2.5	C_8H_{13}
107.01610	0.54	— No match found —				
97.10077	1.81	— No match found —				
95.08457	0.69	— No match found —				
94.00273	0.74	— No match found —				
87.02642	0.61	87.02685	4.9	0.4	1.5	C_4H_7S
85.10082	2.58	— No match found —				
84.09356	5.49	84.09390	4.1	0.3	1.0	C_6H_{12}
83.08588	7.67	83.08607	2.3	0.2	1.5	C_6H_{11}
82.07800	2.10	82.07825	3.0	0.2	2.0	C_6H_{10}
81.07050	1.76	81.07043	-0.9	-0.1	2.5	C_6H_9
79.05526	0.52	— No match found —				
73.02895	0.76	73.02896	0.0	0.0	1.5	$C_3H_5O_2$
72.08942	4.63	— No match found —				
71.08592	61.89	71.08607	2.2	0.2	0.5	C_5H_{11}
70.07761	10.05	— No match found —				
69.07031	23.29	69.07043	1.7	0.1	1.5	C_5H_9
68.06235	2.89	68.06260	3.7	0.2	2.0	C_5H_8
67.05470	3.70	67.05478	1.2	0.1	2.5	C_5H_7
65.01993	2.23	— No match found —				
64.01212	0.54	— No match found —				
61.00936	0.64	— No match found —				
60.02053	0.82	— No match found —				
59.03651	1.16	— No match found —				
58.07326	4.42	— No match found —				
57.06987	100.00	— No match found —				
57.03381	0.52	57.03404	4.0	0.2	1.5	C_3H_5O
56.06050	9.42	— No match found —				
55.05445	29.03	— No match found —				
54.04674	2.15	54.04695	3.9	0.2	2.0	C_4H_6
53.03942	1.84	— No match found —				

High resolution probe mass spectrum for octyl sulfone.

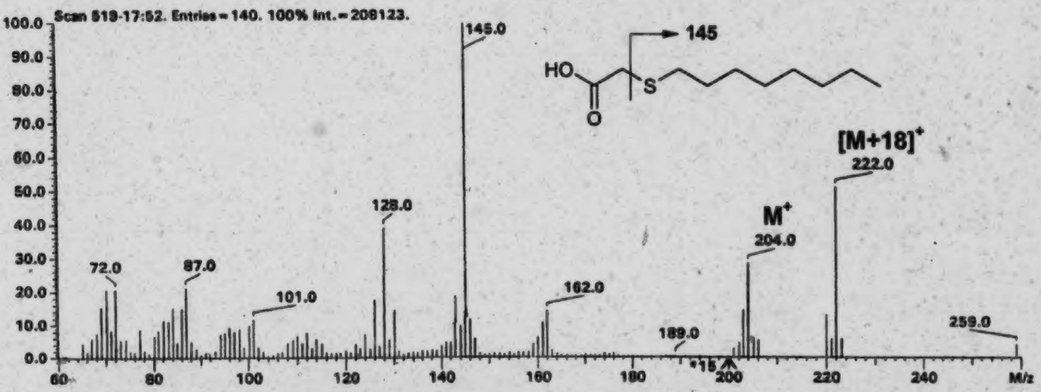


Fourier-transform infrared spectrum for octyl sulfone (GC-FTIR).

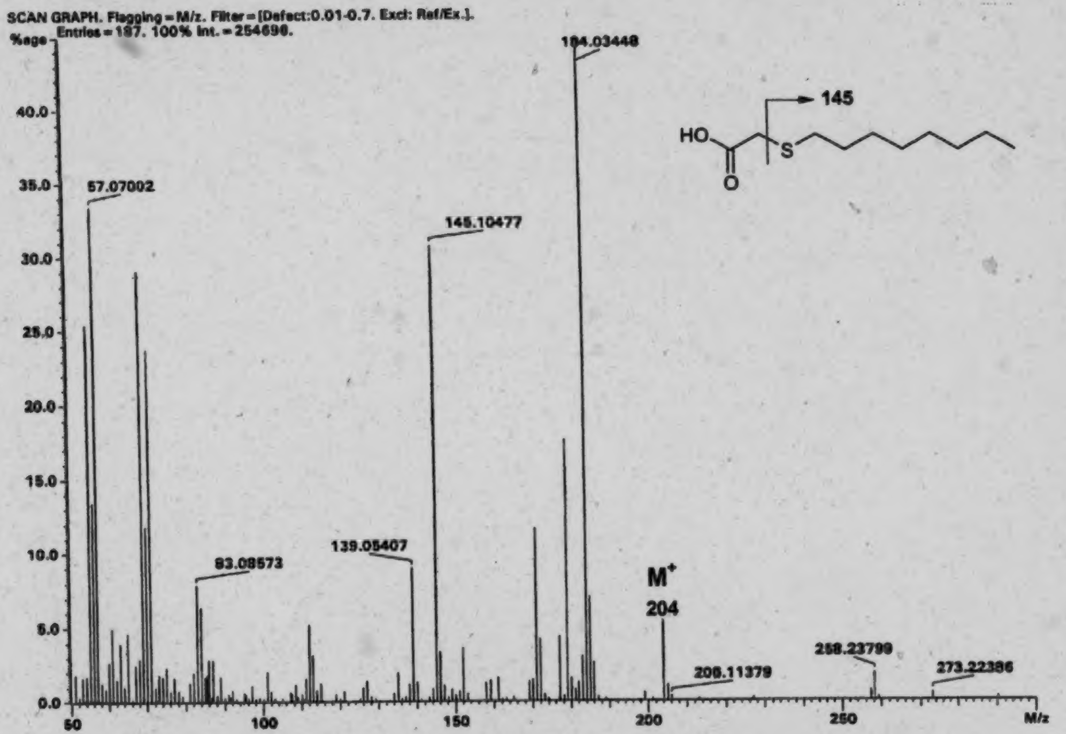
B.2 2-Octylthio acetic acid



Low resolution electron impact mass spectrum for 2-octylthio acetic acid [GC-MS (EI)].



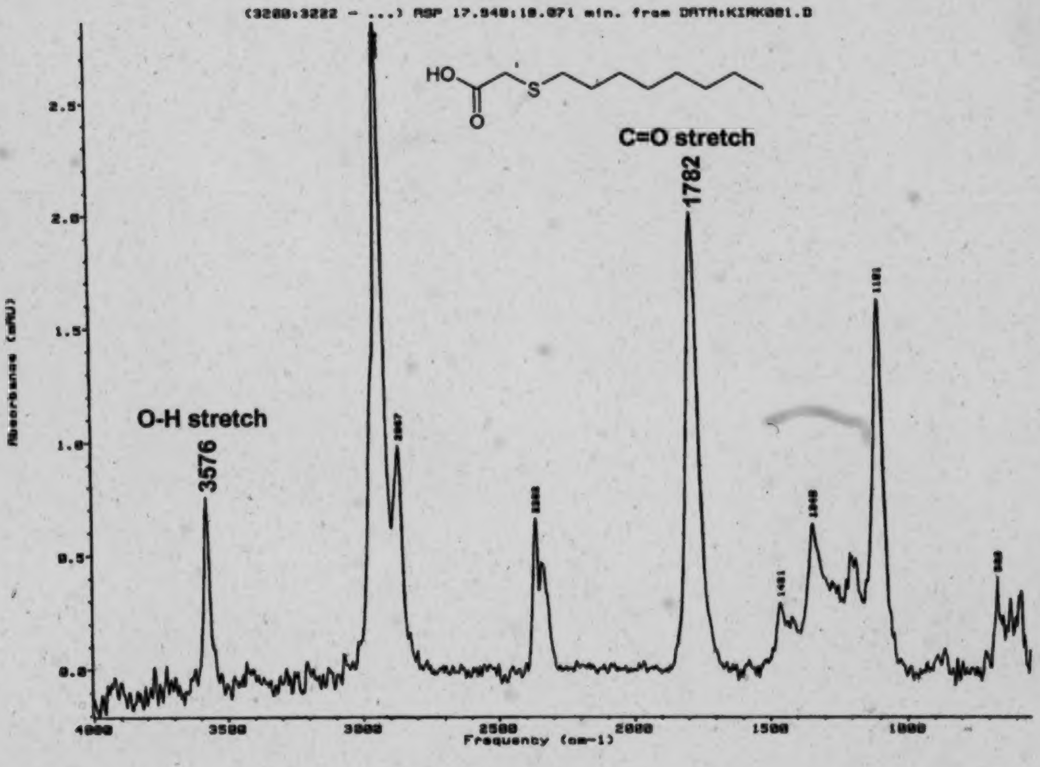
Chemical ionization mass spectrum for 2-octylthio acetic acid [GC-MS (NH_4^+ CI)].



Mass	%age	Calculated	ppm	mmu	R/DB	Formula
258.23799	1.84	258.23813	0.5	0.1	0.0	$C_{16}H_{34}S$
218.00242	10.70	— No match found —				
217.00809	16.07	217.00783	-1.1	-0.2	18.5	$C_{18}H$
216.00621	100.00	216.00674	2.5	0.5	9.0	$C_{12}H_8S_2$
205.12304	1.06	205.12299	-0.3	-0.2	5.5	$C_{13}H_{17}O_2$
204.11763	6.34	204.11841	3.8	0.8	1.0	$C_{10}H_{20}O_2S$
186.03222	2.62	186.03169	-2.8	-0.5	9.0	$C_{11}H_6O_3$
185.03749	7.02	— No match found —				
184.03448	61.29	184.03487	1.0	0.2	9.0	$C_{12}H_8S$
183.02552	3.06	— No match found —				
182.01902	1.32	182.01903	0.0	0.0	10.0	$C_{12}H_6S$
180.11341	1.61	— No match found —				
179.11029	17.58	179.11058	1.7	0.3	-0.5	$C_8H_{19}O_2S$
177.09445	4.37	177.09432	2.7	0.5	0.5	$C_8H_{17}O_2S$
172.03314	4.21	— No match found —				
171.02969	11.63	171.02884	0.8	0.2	8.5	$C_{11}H_7S$
170.01920	1.47	170.01903	4.9	0.8	9.0	$C_{11}H_6S$
169.01044	1.35	169.01120	4.5	0.8	9.5	$C_{11}H_5S$
161.09952	1.57	161.10001	3.0	0.5	0.5	$C_8H_{17}OS$
159.11996	1.40	159.12074	4.9	0.8	0.5	$C_9H_{19}S$
158.01946	1.25	158.01903	3.7	0.6	8.0	$C_{10}H_6S$
152.06234	3.60	152.06261	1.8	0.3	9.0	$C_{12}H_8$
147.11716	1.02	147.11737	1.2	0.2	4.5	$C_{11}H_{15}$
147.10041	1.09	— No match found —				
146.10063	3.33	— No match found —				
145.10477	30.79	145.10510	2.3	0.3	0.5	$C_8H_{17}S$
140.05955	1.31	— No match found —				
139.05407	9.00	— No match found —				
138.04641	1.18	138.04695	3.8	0.5	9.0	$C_{11}H_6$
135.08038	1.86	135.08099	4.5	0.6	4.5	$C_9H_{11}O$
127.05451	1.39	127.05478	2.1	0.3	7.5	$C_{10}H_7$
115.05502	1.24	115.05478	-2.1	-0.2	6.5	C_9H_7
113.13223	3.15	— No match found —				
112.12463	5.10	— No match found —				
111.11671	1.57	— No match found —				
108.50405	1.15	— No match found —				
108.00272	9.97	— No match found —				
101.04101	2.02	— No match found —				
97.10067	1.12	— No match found —				
96.00277	2.47	— No match found —				
89.03984	1.69	— No match found —				
87.02812	2.84	— No match found —				
86.01054	2.88	— No match found —				
85.51256	1.84	— No match found —				
85.10000	1.58	— No match found —				
85.02874	1.70	85.02895	2.6	0.2	2.5	$C_4H_5O_2$
85.00903	1.61	— No match found —				
84.09352	6.38	84.09390	4.5	0.4	1.0	C_6H_{12}
83.08573	7.87	83.08607	4.2	0.3	1.5	C_6H_{11}
82.07791	1.99	82.07825	4.1	0.3	2.0	C_6H_{10}
81.07011	1.31	81.07043	3.9	0.3	2.5	C_6H_9
79.00903	1.07	— No match found —				
77.03902	1.84	77.03912	1.3	0.1	4.5	C_6H_5
77.00633	2.19	77.00611	-2.8	-0.2	0.5	C_2H_5OS
75.02481	2.36	— No match found —				
74.01606	1.70	— No match found —				
73.02878	1.86	73.02895	2.4	0.2	1.5	$C_3H_5O_2$
73.01065	1.50	— No match found —				
72.08836	1.03	— No match found —				
71.08577	23.76	71.08607	4.3	0.3	0.5	C_5H_{11}
70.07741	11.80	— No match found —				
69.07013	29.05	69.07043	4.3	0.3	1.5	C_5H_9
68.06231	2.91	68.06260	4.2	0.3	2.0	C_5H_8
67.05468	2.51	67.05478	1.5	0.1	2.5	C_5H_7
65.02005	4.65	— No match found —				
64.01212	1.03	— No match found —				
63.02332	3.85	63.02348	2.5	0.2	4.5	C_5H_3
62.01584	1.53	62.01565	0.2	0.0	5.0	C_5H_2
61.02942	2.32	— No match found —				
61.01080	4.80	— No match found —				
60.02075	2.68	— No match found —				
60.00270	1.76	— No match found —				
59.07237	1.26	— No match found —				
57.07002	33.42	— No match found —				
57.03380	1.30	57.03404	4.2	0.2	1.5	C_3H_5O
56.06206	13.48	— No match found —				
55.05448	25.40	— No match found —				
55.01815	1.21	55.01839	4.4	0.2	2.5	C_3H_3O
54.04663	1.71	— No match found —				
53.03899	1.66	53.03912	2.5	0.1	2.5	C_4H_5
51.02340	1.83	51.02348	1.5	0.1	3.5	C_4H_3

Page 1

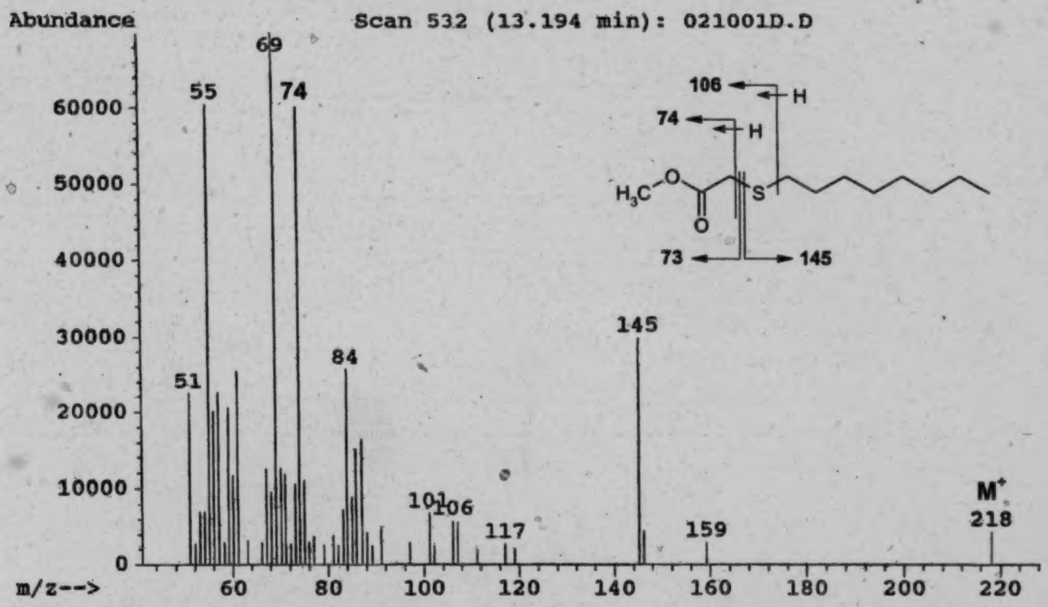
High resolution probe mass spectrum for 2-octylthio acetic acid.



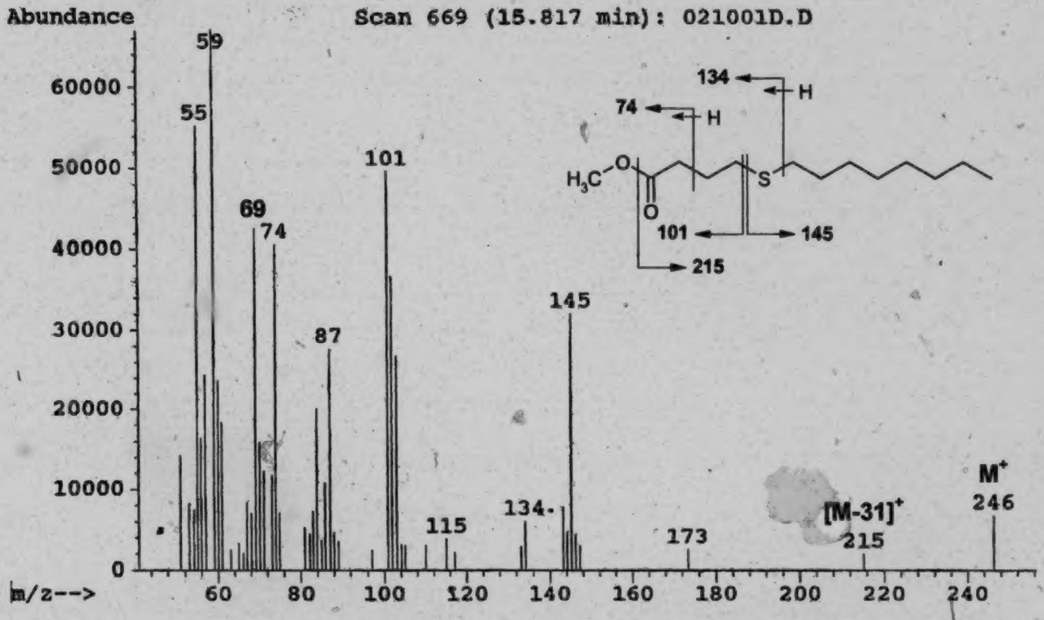
Fourier-transform infrared spectrum for 2-octylthio acetic acid (GC-FTIR).

B.3 Methyl esters of octylthio alkanoic acids

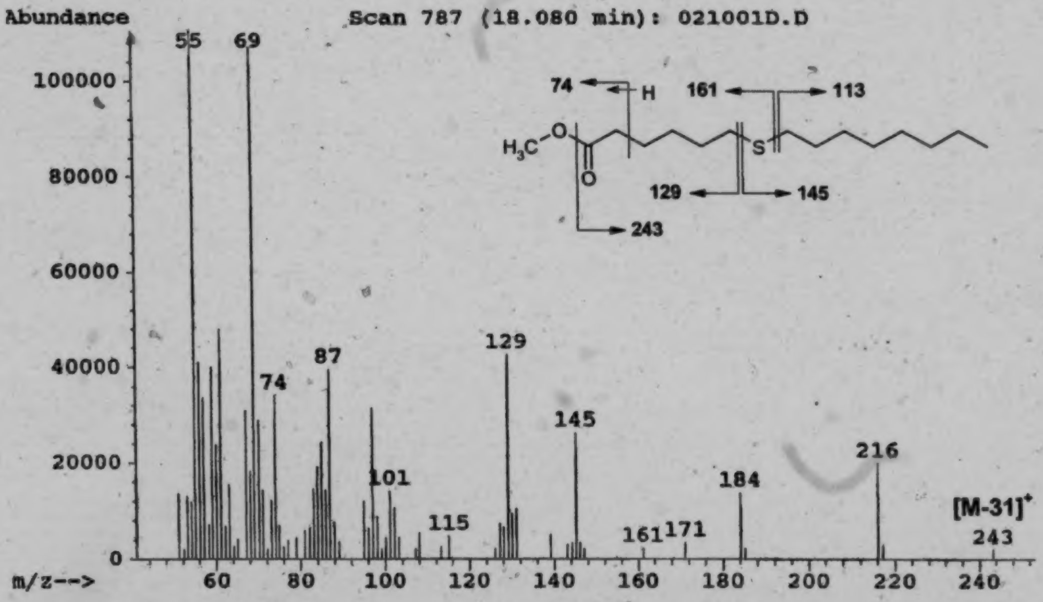
The spectra in this section were obtained by GC-MS (EI).



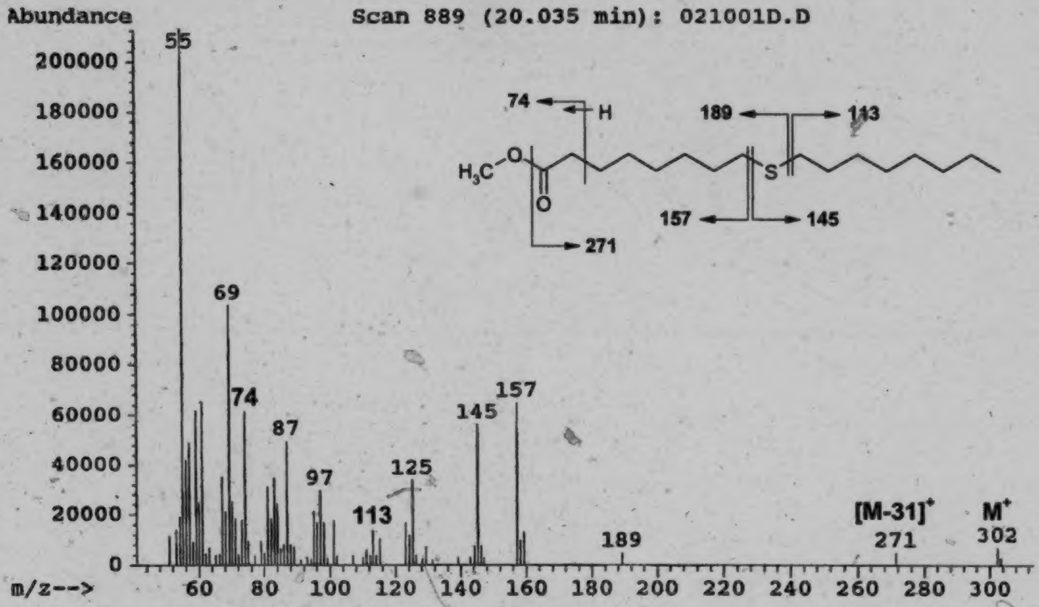
Low resolution mass spectrum for the methyl ester of 2-octylthio acetic acid.



Low resolution mass spectrum for the methyl ester of 4-octylthio butanoic acid.



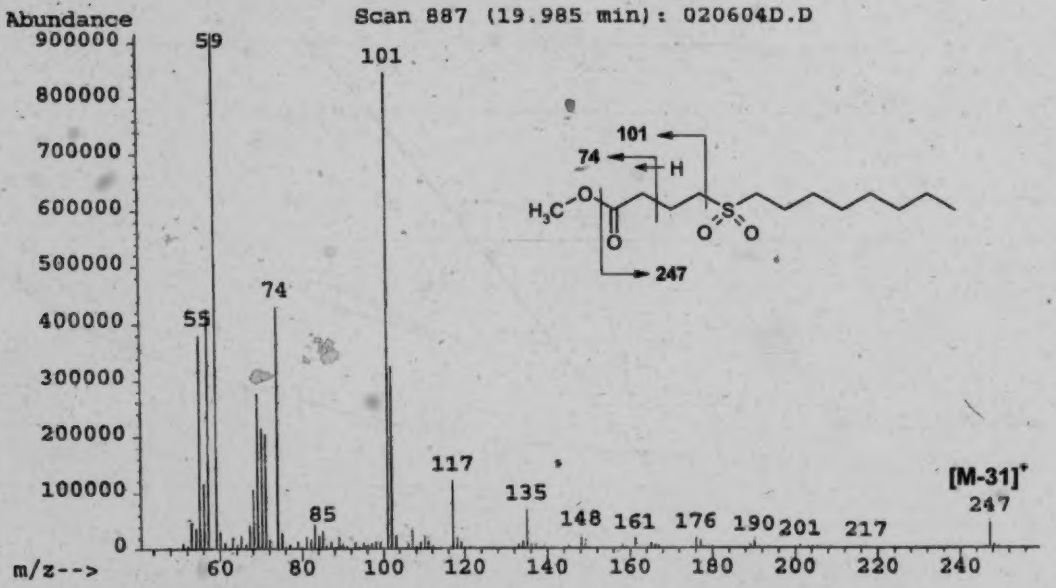
Low resolution mass spectrum for the methyl ester of 6-octylthio hexanoic acid.



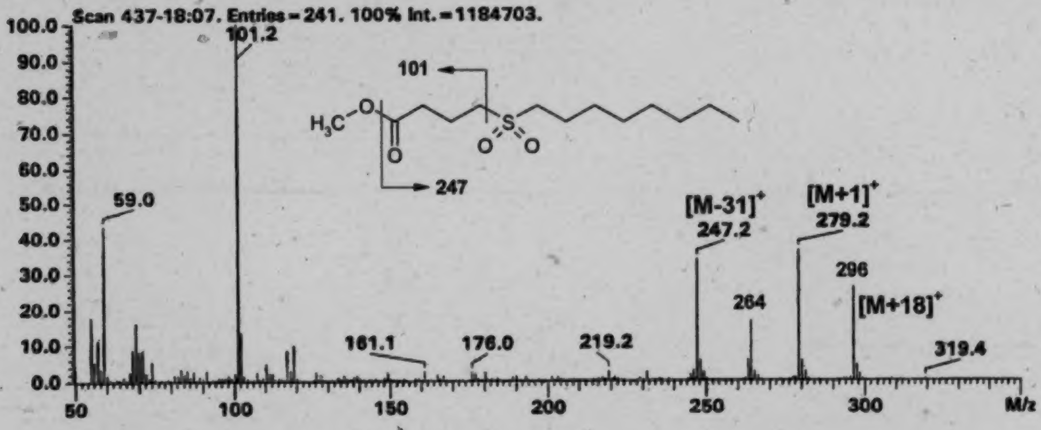
Low resolution mass spectrum for the methyl ester of 8-octylthio octanoic acid.

B.4 Methyl esters of octylsulfonyl alkanoic acids

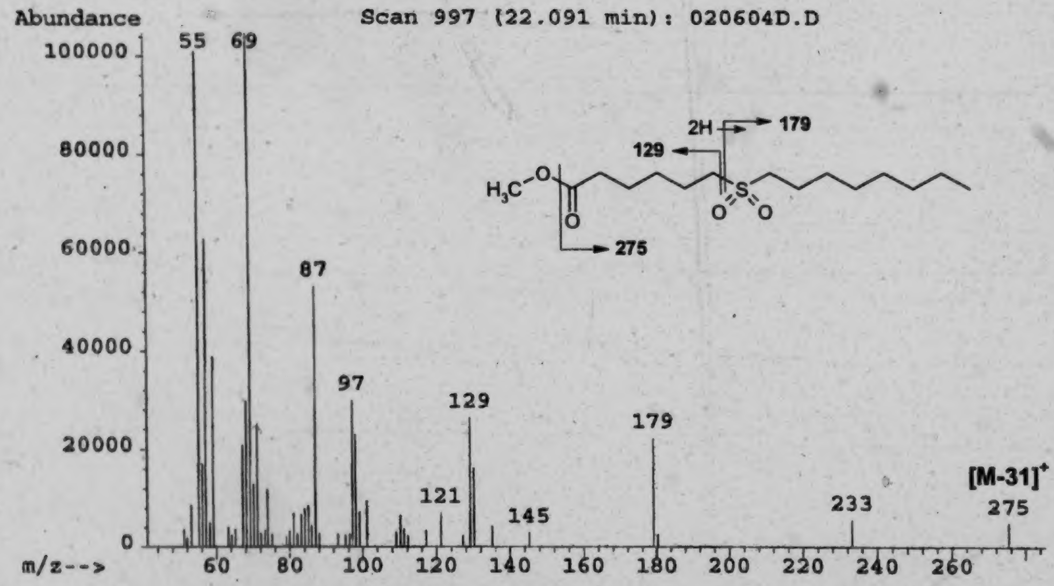
Low resolution mass spectra were obtained by GC-MS (EI). Chemical ionization mass spectra were obtained by GC-MS (NH_4^+ CI).



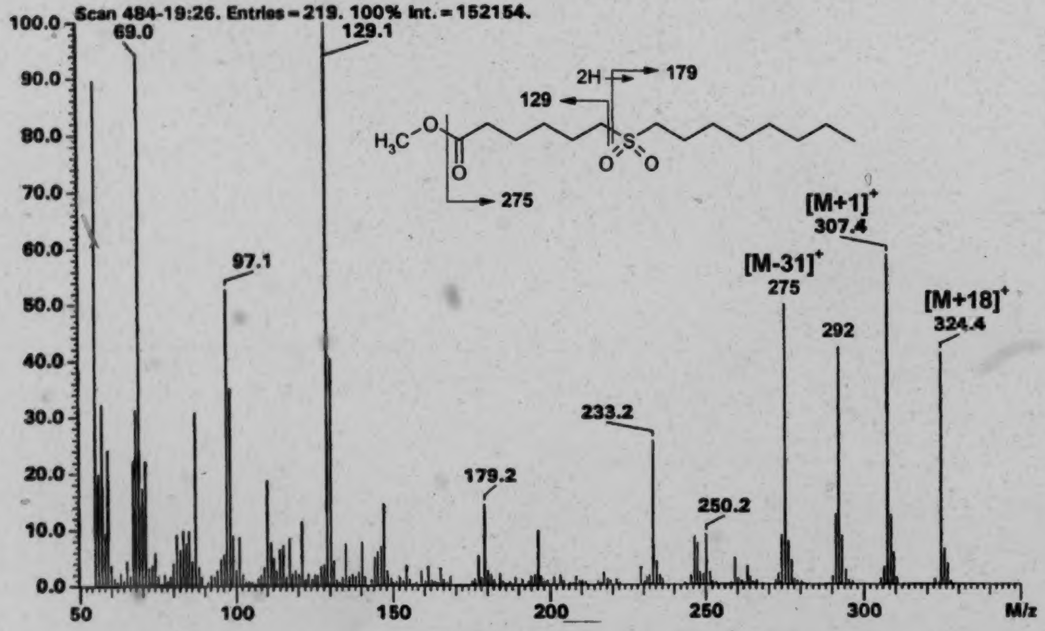
Low resolution mass spectrum for the methyl ester of 4-octylsulfonyl butanoic acid.



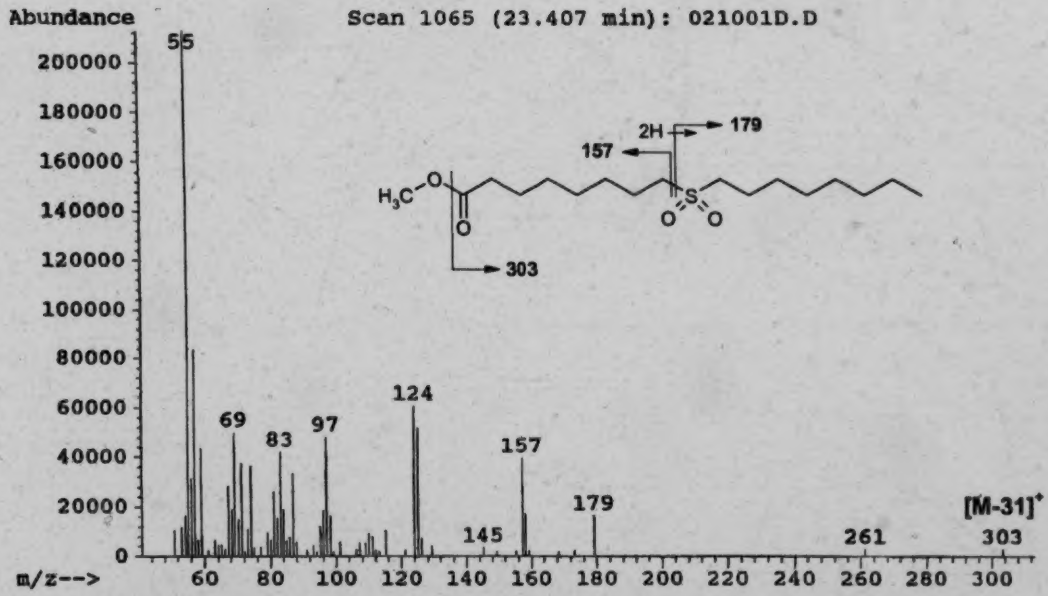
Chemical ionization mass spectrum for the methyl ester of 4-octylsulfonyl butanoic acid.



Low resolution mass spectrum for the methyl ester of 6-octylsulfonyl hexanoic acid.



Chemical ionization mass spectrum for the methyl ester of 6-octylsulfonyl hexanoic acid.



Low resolution mass spectrum for the methyl ester of 8-octylsulfonyl octanoic acid.

Appendix C Raw data, calculations, and statistical analyses for the crude oil experiment

This appendix presents the raw data, calculations, and statistical analyses for yield, viscosity analysis, and sulfur analysis of the oil samples from the crude oil experiment with *Rhodococcus* sp. strain JVH1

C.1 Yield calculations

Initial mass of oil:	25.13 g
Solids removed:	0.1108 g
Total volume of stock in DCM:	500 mL
Stock concentration:	50.0384 g L^{-1}
Volume of stock added per flask:	20 mL
Estimated mass of oil per flask:	1.000768 g

After extraction, all the samples were dried and weighed:

Sample	Jar (g)	Jar + oil (g)	Oil (g)	Yield (%)	Comments
Sterile DBS	85.2885	86.1385	0.8500	84.7	-calc including added DBS
JVH1 DBS	85.5844	86.4158	0.8314	82.9	-calc including added DBS
JVH1 #1	85.8178	86.6426	0.8248	82.4	
JVH1 #2	85.1991	86.0228	0.8237	82.3	
JVH1 #3	85.4674	86.2711	0.8037	80.3	
Sterile #1	85.4928	86.3347	0.8419	84.1	
Sterile #2	85.8732	86.7119	0.8387	83.8	
Sterile #3	85.6254	86.4757	0.8503	85.0	
Killed #1	85.3332	86.1711	0.8379	83.7	-contaminated, not included
Killed #2	85.4677	86.2966	0.8289	82.8	
Killed #3	85.4684	86.2580	0.7896	78.9	

Sample	Average yield (%)	Std Dev
Sterile DBS	84.7	
JVH1 DBS	82.9	
JVH1	81.7	1.19
Sterile	84.3	0.60
Killed	80.9	
Overall	**82.7**	**1.91**

C.2 Viscosity data and statistics

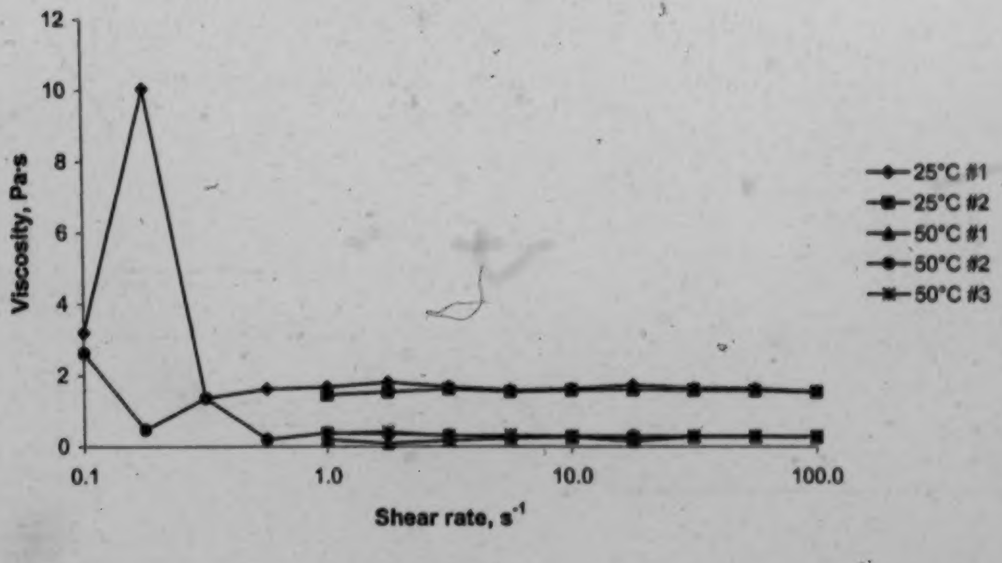
Sample data

Lloydminster crude oil 83-02

Shear rate	1st run		2nd run		1st run		2nd run		3rd run	
rad/s	T, °C	η, Pa·s	T, °C	η, Pa·s	T, °C	η, Pa·s	T, °C	η, Pa·s	T, °C	η, Pa·s
0.10	25.2	3.20					49.7	2.62		
0.18	25.2	10.04					49.7	0.47		
0.32	25.1	1.35					49.7	1.36		
0.56	25.2	1.61					49.6	0.19		
1.00	25.2	1.66	25.2	1.45	49.8	0.19	49.6	0.37	49.7	0.35
1.78	25.1	1.80	25.2	1.53	49.7	0.09	49.6	0.35	49.7	0.40
3.16	25.1	1.67	25.2	1.61	49.7	0.17	49.6	0.31	49.7	0.29
5.62	25.1	1.58	25.2	1.54	49.7	0.23	49.6	0.25	49.7	0.32
10.00	25.1	1.62	25.2	1.58	49.7	0.27	49.6	0.29	49.7	0.28
17.78	25.1	1.72	25.2	1.59	49.7	0.15	49.6	0.29	49.7	0.25
31.62	25.1	1.64	25.2	1.58	49.7	0.27	49.6	0.28	49.7	0.29
56.23	25.1	1.61	25.2	1.56	49.7	0.27	49.7	0.28	49.7	0.29
100.00	25.1	1.54	25.2	1.52	49.7	0.26	49.6	0.28	49.7	0.29

-run #1 at 50°C was not used for further analyses

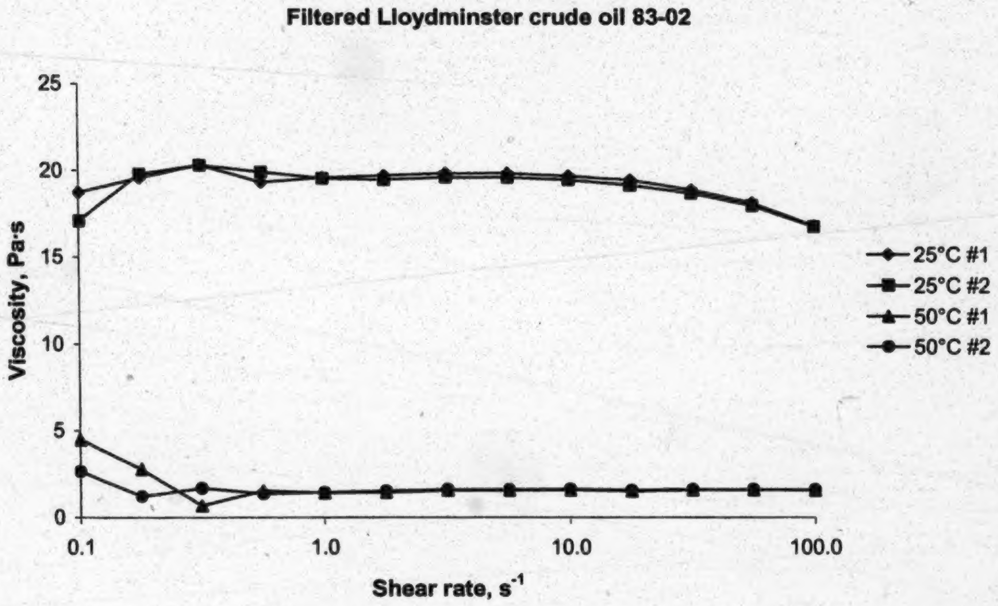
Lloydminster crude oil 83-02



Sample data

Filtered Lloydminster crude oil 83-02

Shear rate	1st run		2nd run		1st run		2nd run	
rad/s	T, °C	η, Pa·s	T, °C	η, Pa·s	T, °C	η, Pa·s	T, °C	η, Pa·s
0.10	25.0	18.73	25.0	17.08	50.4	4.50	50.4	2.66
0.18	25.0	19.57	25.0	19.79	50.4	2.79	50.3	1.22
0.32	25.0	20.31	25.0	20.30	50.4	0.67	50.3	1.69
0.56	25.0	19.31	24.9	19.90	50.4	1.54	50.3	1.36
1.00	25.0	19.58	24.9	19.53	50.4	1.41	50.3	1.45
1.78	25.0	19.68	24.9	19.46	50.4	1.45	50.3	1.53
3.16	25.0	19.83	25.0	19.60	50.4	1.56	50.3	1.62
5.62	25.0	19.81	25.0	19.58	50.4	1.56	50.3	1.61
10.00	25.0	19.65	25.0	19.44	50.4	1.59	50.3	1.63
17.78	25.0	19.42	25.0	19.12	50.4	1.51	50.3	1.58
31.62	25.0	18.85	25.0	18.66	50.4	1.59	50.3	1.62
56.23	25.0	18.09	25.0	17.94	50.4	1.59	50.3	1.62
100.00	25.0	16.77	25.0	16.74	50.4	1.57	50.3	1.60

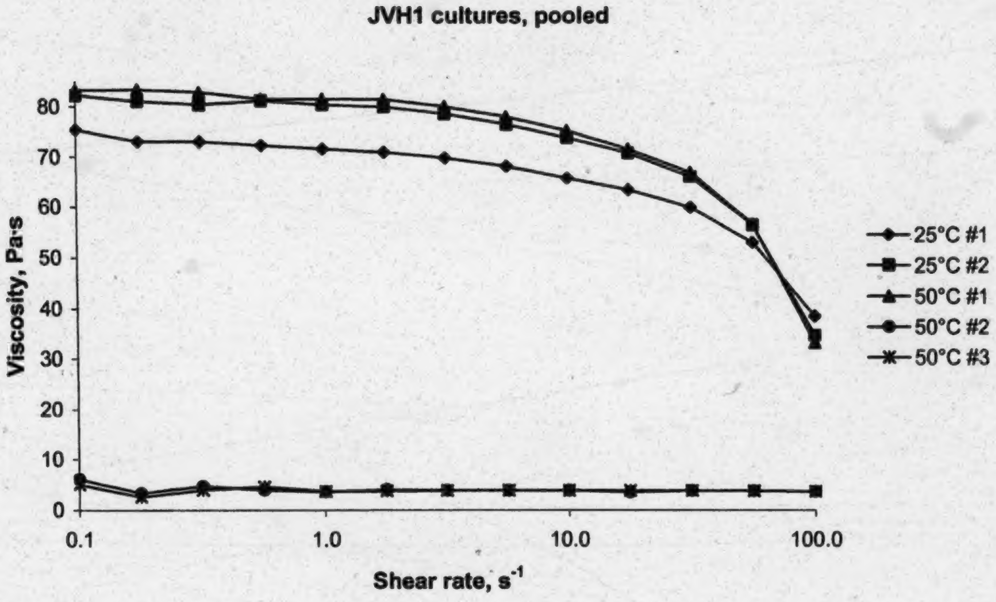


Sample data

JVH1 cultures, pooled

Shear rate	1st run		2nd run		3rd run		1st run		2nd run	
rad/s	T, °C	η, Pa·s	T, °C	η, Pa·s	T, °C	η, Pa·s	T, °C	η, Pa·s	T, °C	η, Pa·s
0.10	24.4	75.40	24.4	82.26	24.5	83.20	50.2	6.08	50.5	5.07
0.18	24.3	73.08	24.4	81.06	24.4	83.37	50.4	3.37	50.5	2.60
0.32	24.2	73.03	24	80.36	24.4	82.85	50.5	4.71	50.5	4.00
0.56	24.2	72.33	24.4	81.15	24.5	81.45	50.5	3.98	50.5	4.62
1.00	24.3	71.58	24.3	80.30	24.4	81.43	50.5	3.68	50.5	3.71
1.78	24.3	70.87	24.3	79.89	24.4	81.36	50.5	4.01	50.5	3.73
3.16	24.3	69.76	24.3	78.46	24.4	79.92	50.4	3.88	50.5	3.87
5.62	24.3	68.06	24.4	76.43	24.4	77.91	50.4	3.89	50.5	3.91
10.00	24.3	65.71	24.4	73.81	24.4	75.16	50.4	3.89	50.5	3.87
17.78	24.3	63.36	24.3	70.75	24.4	71.52	50.4	3.54	50.5	3.83
31.62	24.3	59.88	24.3	66.03	24.4	66.87	50.5	3.84	50.5	3.84
56.23	24.3	52.99	24.3	56.45	24.4	56.56	50.5	3.79	50.4	3.80
100.00	24.3	38.51	24.3	34.67	24.4	33.17	50.5	3.69	50.5	3.69

-run #1 at 25°C was not used for further analyses



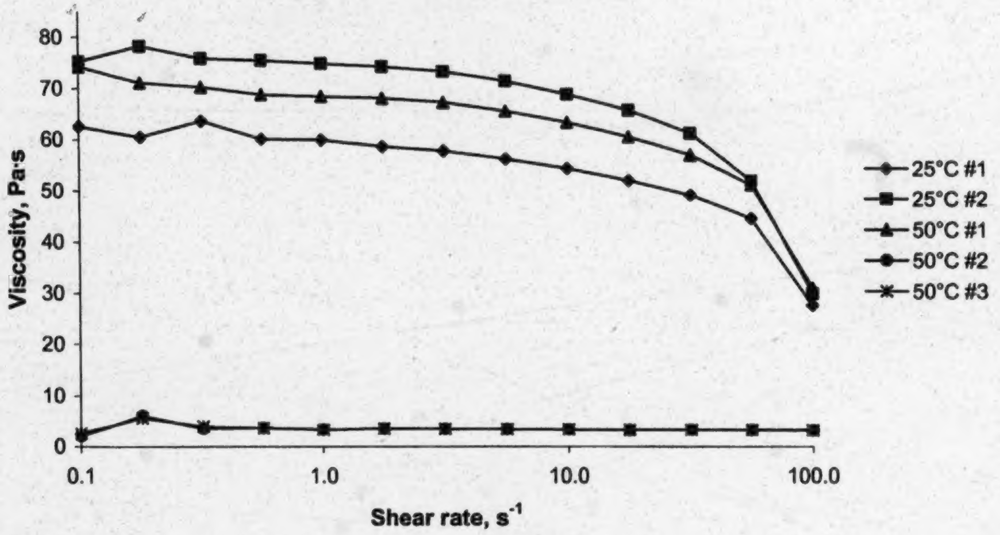
Sample data

JVH1 killed cell controls, pooled

Shear rate	1st run		2nd run		3rd run		1st run		2nd run	
rad/s	T, °C	η, Pa·s	T, °C	η, Pa·s	T, °C	η, Pa·s	T, °C	η, Pa·s	T, °C	η, Pa·s
0.10	25.2	62.55	24.5	75.24	24.5	74.24	50.4	2.04	50.6	2.639
0.18	25.2	60.50	24.4	78.24	24.4	71.14	50.5	6.03	50.6	5.623
0.32	25.2	63.61	24.4	75.87	24.4	70.31	50.6	3.55	50.6	4.018
0.56	25.2	60.13	24.4	75.50	24.4	68.83	50.6	3.72	50.6	3.668
1.00	25.3	59.91	24.4	74.89	24.4	68.45	50.6	3.37	50.6	3.30
1.78	25.3	58.66	24.4	74.27	24.4	68.12	50.6	3.51	50.6	3.55
3.16	25.3	57.79	24.4	73.33	24.4	67.25	50.6	3.44	50.6	3.46
5.62	25.3	56.23	24.4	71.46	24.4	65.57	50.6	3.41	50.6	3.41
10.00	25.3	54.35	24.4	68.89	24.5	63.37	50.6	3.38	50.6	3.37
17.78	25.2	52.01	24.4	65.75	24.4	60.56	50.6	3.24	50.6	3.27
31.62	25.3	49.17	24.4	61.30	24.4	57.06	50.6	3.31	50.6	3.33
56.23	25.3	44.54	24.4	52.01	24.4	51.18	50.6	3.27	50.5	3.29
100.00	25.3	27.62	24.4	29.98	24.4	31.01	50.6	3.17	50.5	3.20

-run #1 at 25°C was not used for further analyses

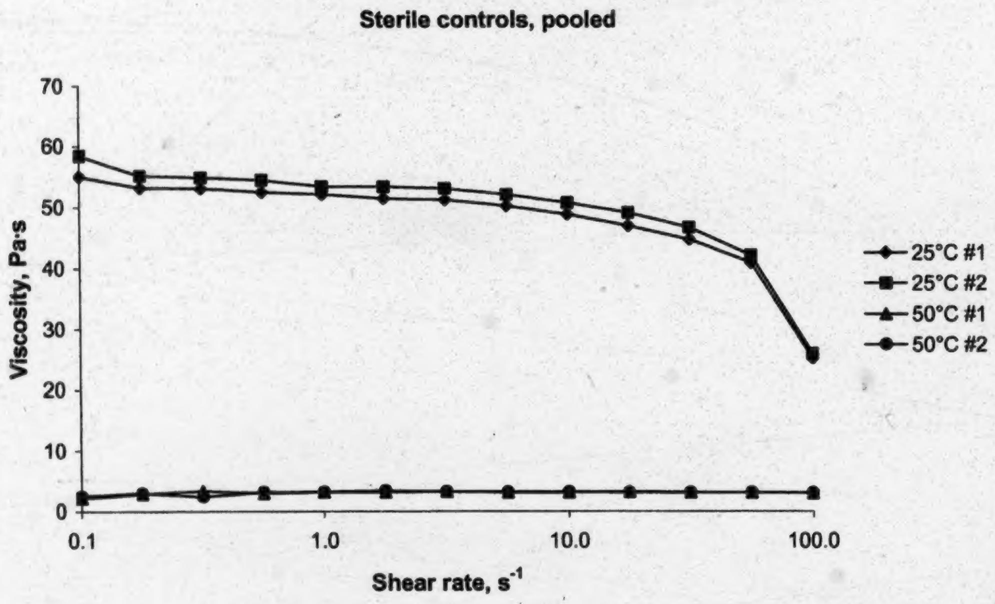
JVH1 killed cell controls, pooled



Sample data

Sterile controls, pooled

Shear rate	1st run		2nd run		1st run		2nd run	
rad/s	T, °C	η, Pa·s	T, °C	η, Pa·s	T, °C	η, Pa·s	T, °C	η, Pa·s
0.10	24.4	55.04	24.5	58.44	50.0	2.18	49.7	2.49
0.18	24.4	53.22	24.4	55.20	49.8	2.85	49.7	3.01
0.32	24.4	53.09	24.4	54.89	49.7	3.34	49.7	2.43
0.56	24.4	52.49	24.4	54.46	49.7	3.00	49.7	3.21
1.00	24.4	52.12	24.4	53.40	49.7	3.15	49.7	3.18
1.78	24.4	51.43	24.4	53.32	49.7	3.16	49.7	3.41
3.16	24.4	51.15	24.4	53.04	49.7	3.25	49.6	3.22
5.62	24.4	50.17	24.4	52.06	49.7	3.13	49.7	3.18
10.00	24.4	48.77	24.4	50.69	49.7	3.11	49.7	3.18
17.78	24.4	46.90	24.4	49.05	49.7	3.20	49.7	3.07
31.62	24.4	44.67	24.4	46.61	49.7	3.10	49.7	3.13
56.23	24.4	40.90	24.4	42.06	49.7	3.07	49.6	3.11
100.00	24.4	25.10	24.4	25.88	49.7	3.00	49.7	3.04



Statistical analysis of extracts - ANOVA at 25°C and 50°C

	Viscosity at 25°C, Pa·s			Viscosity at 50°C, Pa·s		
	JVH1	Killed	Sterile	JVH1	Killed	Sterile
1st run	80.30	74.89	52.12	3.68	3.37	3.15
	79.89	74.27	51.43	4.01	3.51	3.16
	78.46	73.33	51.15	3.88	3.44	3.25
	76.43	71.46	50.17	3.89	3.41	3.13
	73.81	68.89	48.77	3.89	3.38	3.11
2nd run	81.43	68.45	53.40	3.71	3.30	3.18
	81.36	68.12	53.32	3.73	3.55	3.41
	79.92	67.25	53.04	3.87	3.46	3.22
	77.91	65.57	52.06	3.91	3.41	3.18
	75.16	63.37	50.69	3.87	3.37	3.18

Anova: Single Factor at 25°C

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
JVH1	10	784.7	78.47	6.859
Killed	10	695.6	69.56	14.638
Sterile	10	516.2	51.62	2.207

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatments	3741	2	1871	237	7.6E-18	3.35
Residuals	213.3	27	7.901			
Total	3955	29				

Anova: Single Factor at 50°C

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
JVH1	10	38.4	3.84	0.011
Killed	10	34.2	3.42	0.005
Sterile	10	32.0	3.20	0.007

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.154	2	1.077	140	5.8E-15	3.35
Within Groups	0.2084	27	0.007720			
Total	2.362	29				

Statistical analysis of extracts - Tukey's test for multiple paired comparisons, equal groups

Sample means	JVH1	Killed	Sterile
25°C	78.47	69.56	51.62
50°C	3.84	3.42	3.20

	25°C	50°C	
α	0.05	0.05	
error mean square (s^2)	7.901	0.007720	
degrees of freedom (df)	27	27	
treatments (k)	3	3	
observations (n)	10	10	
q(α, k, df)	3.51	3.51	*-Studentized range statistic*
HSD	3.12	0.0975	

Honestly significant difference:

$$HSD = q\left[\alpha, k, df\right]\sqrt{\frac{s^2}{n}}$$

At 25°C	Differences		Significant?	
	Killed	Sterile	Killed	Sterile
JVH1	8.907	26.852	**yes**	**yes**
Killed		17.95		**yes**

At 50°C	Differences		Significant?	
	Killed	Sterile	Killed	Sterile
JVH1	0.424	0.646	**yes**	**yes**
Killed		0.223		**yes**

Summary:

At both 25°C and 50°C, all three samples are significantly different from each other. The viscosity is highest for the live JVH1 cultures, and lowest for the cell-free sterile controls.

C.3 Sulfur data, calculations, and statistics

Standard curve - Regression analysis (Method of least squares)

Constants

number of points = $n = 9$

$$D = n\sum\left(x_i^{\,2}\right) - \left(\sum x_i\right)^2$$

Slope

$$m = \frac{n\sum\left(x_i y_i\right) - \sum x_i \sum y_i}{D}$$

Intercept

$$b = \frac{\sum\left(x_i^{\,2}\right)\sum y_i - \sum x_i \sum\left(x_i y_i\right)}{D}$$

Residuals

$$d_i = y_i - \left(m x_i + b\right)$$

Uncertainty in the regression parameters

Standard deviation of the residuals

$$\sigma_y \approx s_y = \sqrt{\frac{\sum\left(d_i^{\,2}\right)}{n-2}}$$

Standard deviation of the slope

$$\sigma_m = \sigma_y\sqrt{\frac{n}{D}}$$

Standard deviation of the intercept

$$\sigma_b = \sigma_y\sqrt{\frac{\sum\left(x_i^{\,2}\right)}{D}}$$

Calculations of concentration using the regression parameters

Constants

average peak area = y

number of peak area values averaged = $k = 3$

Concentration and standard deviation of sulfur in the oil/DCM solution in mg L^{-1}

$$c_{\text{sulfur, DCM + oil}} = \frac{y-b}{m}$$

$$\sigma_{\text{c,DCM}} = \frac{\sigma_y}{m}\sqrt{\frac{1}{k} + \left(\frac{y-b}{m}\right)^2\left(\frac{n}{D}\right) + \frac{\sum\left(x_i^{\,2}\right)}{D} - 2\left(\frac{y-b}{m}\right)\left(\frac{\sum x_i}{D}\right)}$$

Concentration and standard deviation of sulfur in the oil as % wt/wt (neglecting error in m_{oil})

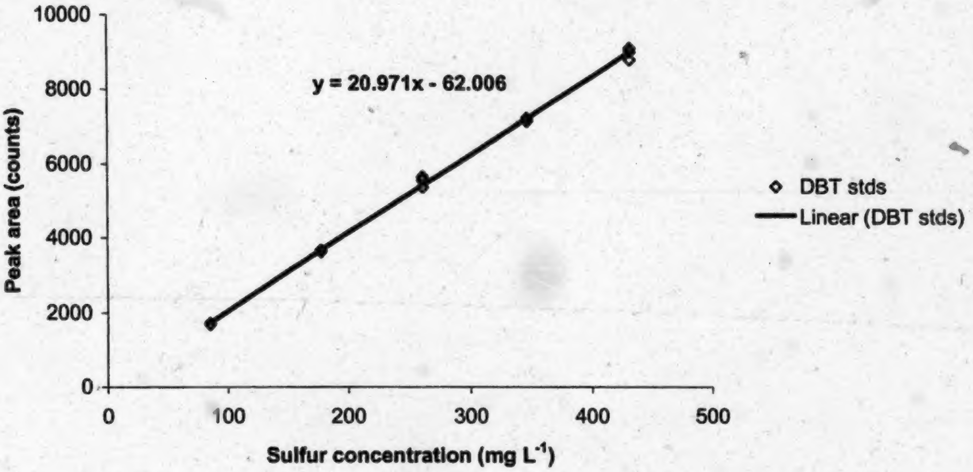
$$c_{\text{sulfur, oil}} = \frac{(0.01)\left(c_{\text{sulfur, DCM + oil}}\right)}{m_{\text{oil}}} \times 100\%$$

$$\sigma_{\text{c,oil}} = \sigma_{\text{c,DCM}}\,\frac{c_{\text{sulfur, oil}}}{c_{\text{sulfur, DCM + oil}}}$$

Dibenzothiophene standards in 10 mL DCM

m_{DBT} (mg)	c_{sulfur} (mg S L^{-1})	Peak area (counts)	Regression calculations		Residuals	
	x_i	y_i	$x_i y_i$	$(x_i)^2$	d_i	$(d_i)^2$
4.9	85	1710	145722	7262	-15.1	227
	85	1679	143080	7262	-46.1	2121
	85	1701	144955	7262	-24.1	579
10.2	177	3647	646946	31468	-11.0	121
	177	3612	640737	31468	-46.0	2116
	177	3650	647478	31468	-8.0	64
15.0	261	5639	1471043	68053	230.4	53090
	261	5535	1443913	68053	126.4	15980
	261	5355	1396957	68053	-53.6	2872
19.9	346	7203	2492864	119776	7.4	54
	346	7191	2488711	119776	-4.6	22
	346	7126	2466216	119776	-69.6	4851
24.8	431	8782	3787715	186023	-200.7	40285
	431	9088	3919694	186023	105.3	11086
	431	8992	3878289	186023	9.3	86
Sums:	3903	80910	25714320	1237747	0	133552

	$D=$	3335846	Std dev of residuals:	$\sigma_y \sim s_y =$	101.4
Slope:	$m=$	20.97	Std dev of slope:	$\sigma_m =$	0.215
Intercept:	$b=$	-62.0	Std dev of intercept:	$\sigma_b =$	61.7



Individual samples

Sample	m_{oil}	Peak area (counts)				$c_{sulfur,DCM+oil}$		$c_{sulfur,oil}$	
	(mg)	Inj #1	Inj #2	Inj #3	Average	(mg L^{-1})	$\sigma_{c,DCM}$	(% wt/wt)	$\sigma_{c,oil}$
83-02 oil	109.3	6609	6629	6407	6548	315	2.34	2.88	0.021
83-02 filtered	98.6	6517	6673	6639	6610	318	2.33	3.23	0.024
Sterile #1	96.3	6546	6457	6773	6592	317	2.34	3.29	0.024
Sterile #2	95.1	6602	6682	6789	6691	322	2.32	3.39	0.024
Sterile #3	100.1	7055	7147	7264	7155	344	2.26	3.44	0.023
Killed #1	96.1	6916	7066	6831	6938	334	2.29	3.47	0.024
Killed #2	101.7	7419	7218	7216	7284	350	2.25	3.44	0.022
Killed #3	107.3	7418	7337	7468	7408	356	2.23	3.32	0.021
	-Killed #1 was contaminated and is not included in further calculations								
JVH1 #1	93.8	6680	6742	6687	6703	323	2.32	3.44	0.025
JVH1 #2	116.0	8304	8389	8201	8298	399	2.16	3.44	0.019
JVH1 #3	105.1	7453	7759	7724	7645	368	2.21	3.50	0.021
Sterile DBS	92.0	6672	6590	6474	6579	317	2.34	3.44	0.025
JVH1 DBS	91.0	6820	6850	6819	6830	329	2.30	3.61	0.025

Weighted average concentrations

Weight

$$w_i = \frac{1}{\left(\sigma_{c,oil}\right)_i^2}$$

Weighted average concentration and standard deviation of sulfur in the oil as % wt/wt

$$c_{\text{sulfur, oil, weighted avg}} = \frac{\sum (w_i c_i)}{\sum w_i}$$

$$\sigma_{\text{c,oil, wt avg}} = \frac{1}{\sqrt{\sum w_i}}$$

Weighted averages for each condition

Sample	Weighted average calculations						$c_{sulfur,oil,weighted\ avg}$	
	w_1	w_1c_1	w_2	w_2c_2	w_3	w_3c_3	(% wt/wt)	$\sigma_{c,oil,wt\ avg}$
83-02 oil	2177	6278					2.88	0.021
83-02 filtered	1786	5762					3.23	0.024
Sterile	1699	5600	1678	5683	1962	6744	3.38	0.014
Killed			2052	7067	2311	7672	3.38	0.015
JVH1	1635	5623	2875	9879	2264	7917	3.46	0.012
Sterile DBS	1548	5330					3.44	0.025
JVH1 DBS	1563	5644					3.61	0.025

Statistical analysis of extracts - ANOVA

Sample	Concentration in DCM (mg L^{-1})								
	Extract #1			Extract #2			Extract #3		
	Inj #1	Inj #2	Inj #3	Inj #1	Inj #2	Inj #3	Inj #1	Inj #2	Inj #3
Sterile	315	311	326	318	322	327	339	344	349
Killed				357	347	347	357	353	359
JVH1	321	324	322	399	403	394	358	373	371

Sample	Concentration in oil (% wt/wt)								
	Extract #1			Extract #2			Extract #3		
	Inj #1	Inj #2	Inj #3	Inj #1	Inj #2	Inj #3	Inj #1	Inj #2	Inj #3
Sterile	3.27	3.23	3.38	3.34	3.38	3.44	3.39	3.43	3.49
Killed				3.51	3.41	3.41	3.32	3.29	3.35
JVH1	3.43	3.46	3.43	3.44	3.47	3.40	3.41	3.55	3.53

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Sterile	9	30.36	3.37	0.0067
Killed	6	20.29	3.38	0.0062
JVH1	9	31.12	3.46	0.0028

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*	*Sign.?*
Treatments	0.0371	2	0.0186	3.63	0.044	3.47	**yes**
Residuals	0.1072	21	0.0051				
Total	0.1443	23					

Summary:

There are significant differences in sulfur content among the samples, at a confidence level of 95%.

Statistical analysis of extracts - Tukey-Kramer test for multiple paired comparisons, unequal groups

	JVH1	Killed	Sterile
Number of samples (*n*)	9	6	9
Sample means	3.46	3.38	3.37

α	0.05
error mean square (s^2)	0.0051
degrees of freedom (*df*)	21
treatments (*k*)	3
q(α, *k*, *df*)	3.56 *-Studentized range statistic*

Honestly significant difference :

$$HSD = q[\alpha, k, df]\sqrt{\frac{s^2}{2}\left(\frac{1}{n_i} + \frac{1}{n_j}\right)}$$

	HSD		Differences		Significant?	
	Killed	Sterile	Killed	Sterile	Killed	Sterile
JVH1	0.0948	0.0848	0.0755	0.0845	**no**	**no**
Killed		0.0948		0.0090		**no**

Summary:
Although the ANOVA indicated significant differences among the samples,
the Tukey-Kramer test did not show significant differences between any of the sample pairs.

Note that the *p*-value for the ANOVA was very close to 0.05 (p=0.044), and that the difference between JVH1 and the sterile control (0.0845) was very close to the *HSD* (0.0848).

C.4 References

Harris, D. C. 1991. Quantitative Chemical Analysis, 3rd ed. W. H. Freeman and Company, New York NY.

Taylor, J. R. 1997. An Introduction to Error Analysis, 2nd ed. University Science Books, Sausalito CA.